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CLINICAL AND GENETIC FINDINGS IN EARLY-ONSET PRIMARY OSTEOPOROSIS

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ACADEMIC DISSERTATION

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To my family

CONTENTS

ABSTRACT

Osteoporosis is a common disease, which is caused by both genetic and environmental factors. Osteoporosis increases the risk of fragility fractures, reduces quality of life, and raises mortality rates in affected individuals. Despite years of extensive genetic research, only a few clinically significant underlying genes have been identified. These have been discovered by linkage analysis of families with extreme bone phenotypes. The numerous gene polymorphisms identified by genome-wide association studies have only modest effects on bone mineral density. This suggests that the osteoporosis phenotype seen in the general population is probably due to complex inheritance, i.e. caused by many interacting genetic factors.

The aim of this investigation was to examine young patients and families with early-onset osteoporosis in order to better define their clinical phenotype and to clarify their genetic predisposition. A cohort of 27 children with juvenile osteoporosis and 60 family members were assessed at the Hospital for Sick Children, Toronto, Canada. The majority of assessed children had at least one parent with low bone mineral density implying a strong heredity of the disorder. Certain polymorphisms (Q89R, V667M, N740N and A1330V) in the gene encoding for low density lipoprotein receptor-related protein 5 (*LRP5*) were shown to be over-represented in this cohort.

Patients with a clinical diagnosis of osteoporosis-pseudoglioma syndrome (OPPG), a rare disease caused by biallelic mutations in *LRP5,* were screened and presented four new mutations affecting protein splicing along with the first functional data on a splice site mutation in *LRP5.*

Finnish families with many individuals suffering from osteoporosis were also assessed. In a family with early-onset osteoporosis, 11 of 19 examined family members were found to be affected. The clinical characteristics include suboptimal bone accrual in adolescence, severe osteoporosis in adulthood, progressive compression fractures of the vertebrae, and proneness to peripheral fractures. Histomorphometric analyses from four affected individuals also indicated low-turnover bone metabolism. Two genomic regions (1p22.2-p21.1 and 11q22.1-q22.3), which may contain their disease-causing genetic variations, were identified by microsatellite linkage analysis. The exons in the putative linkage areas were sequenced by target enrichment and next-generation sequencing, and three heterozygous non-synonymous variations were found in the genes encoding for BCAR3 (p.F6Y), MMP10 (p.R53K), and DYNC2H1 (p.Q304L). These require further assessment to clarify their clinical significance. In another family, the rare syndrome of *Calvarial Doughnut Lesions* (CDL) was diagnosed in a young girl with osteoporosis and severe glaucoma. Her father and paternal grandmother were also affected. A thorough characterization of

the clinical and bone histomorphometric features of the condition is provided here. A role for *LRP5* has been excluded in the pathogenesis of CDL, given that no mutations have been found in this gene to correspond with the clinical cases studied.

This thesis provides new data regarding the clinical features and genetic background of bone weakness in children. The impact of these discovered genetic defects requires further elucidation in future studies.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals.

I. Laine CM, Wessman M, Kaunisto M, Toiviainen-Salo S, Mäyränpää MK, Laine T, Lehesjoki AE, Mäkitie O. New genetic loci linked to early-onset osteoporosis in a large Finnish family. (submitted)

II. Laine CM, Chung BD, Susic M, Prescott T, Semler O, Fiskerstrand T, D'Eufemia P, Castori M, Pekkinen M, Sochett E, Cole WG, Netzer C, Mäkitie O. Novel mutations affecting LRP5 splicing in patients with osteoporosis-pseudoglioma syndrome (OPPG). Eur J Hum Genet. 2011 Aug;19(8):875-81.

III. Laine CM, Koltin D, Susic M, Varley TL, Daneman A, Moineddin R, Cole WG, Mäkitie O, Sochett E. Primary osteoporosis without features of OI in children and adolescents: Clinical and genetic characteristics. Am J Med Genet A. 2012 Jun;158A(6):1252-61.

IV. Jaakkola E, Laine CM, Mäyränpää MK, Falck A, Ignatius J, Mäkitie O. Calvarial doughnut lesions and osteoporosis: a new three-generation family and review. Am J Med Genet A. 2009 Nov;149A(11):2371-7.

ABBREVIATIONS

A	adenosine
aBMD	areal bone mineral density
ALP	alkaline phosphatase
APC	adenomatous polyposis coli
BCAR3	breast cancer anti-estrogen resistance 3
BMD	bone mineral density
BMP2	bone morphogenetic protein 2
bp	base pairs
C	Cytosine
Ca2+ or Ca	calcium
CDL	calvarial doughnut lesions
cDNA	complementary DNA
CEPH	Centre d'Etude du Polymorphisme Humain
CM	conditioned medium
CNV	copy-number variation
COL1A1	collagen type 1, alpha 1
COL1A2	collagen type 1, alpha 1
CpG	cytosine-phosphate-guanine
CSNK1A1L	casein kinase alpha 1-like
CT	computed tomography
DDK1	dickkopf 1
DNA	deoxyribonucleic acid
DPD	deoxypyridinoline
Dvl	dishevelled
DXA	dual-energy X-ray absorptiometry
ER	endoplasmatic reticulum
FIMM	Institute for Molecular Medicine Finland
Fzd	frizzled receptor
G	guanine
GSK	glycogen synthase kinase
GTF3A	general transcription factor IIIA
GWAS	genome-wide association study
GWMS	genome-wide microsatellite study
HEK293T	human embryonic kidney cell-line 293T
ICTP	C-terminal telopeptide of type I collagen
IGF-1	insulin-like growth factor 1
IJO	idiopathic juvenile osteoporosis
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LEF-1	lymphoid enhancer factor-1
LOD	logarithmic odds
LRP	low density lipoprotein receptor-related protein

LS	lumbar spine
LY	cell lysate
MesdC2	mesoderm development candidate 2
mRNA	messenger ribonucleic acid
MRI	magnetic resonance imaging
NCBI	National Center for Biotechnology Information
NHLBI	National Heart, Lung and Blood Institute
NTX	N-telopeptide of type I collagen
OB	osteblast
OC	osteoclast
OCN	osteocalcin
OI	osteogenesis imperfecta
OPG	osteoprotegerin
OPPG	osteoporosis-pseudoglioma syndrome
PCR	polymerase chain reaction
PICP	procollagen type I C-terminal peptide
PINP	procollagen type I N-terminal peptide
PKC	protein kinase C
PTH	parathyroid hormone
PTHLH	parathyroid hormone-like hormone
qCT	quantitative computed tomography
QUS	quantitative ultrasound
RANK	the receptor activator of nuclear factor-kappaB
RANKL	the receptor activator of nuclear factor-kappaB ligand
RAP	receptor-associated protein
ROA	regulation-of-autophagy
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SNP	single nucleotide polymorphism
SP7	Sp7 transcription factor (also called Osterix)
T	thymine
TCF	T-cell factor
TRAP-5b	Tartrate-resistant acid phosphatase isoform 5b
UCSC	University of California Santa Cruz
vBMD	volumetric bone mineral density
VCF	vertebral compression fracture
WHO	the World Health Organization
WIF	Wnt inhibitory factor
Wnt	Wingless-type (name of pathway and group of ligands)
WT	wild type

1. INTRODUCTION

Osteoporosis is a genetic or acquired bone disorder, which has serious consequences for both affected patients and the community. Osteoporosis-related bone fragility causes significant morbidity, mortality, and increased health care expenditures. The most common forms of osteoporosis are age- and sex-hormone related, or iatrogenic e.g. due to corticosteroid treatment. In patients without obvious external causes of bone weakness, hereditary factors play a significant role: the heredity of osteoporosis between first-degree relatives is high (Stewart & Ralston, 2000). Early identification of patients at risk is crucial. Potent drugs for the prevention of fragility fractures and related complications exist, but the challenge is to diagnose the bone weakness before it becomes symptomatic and to choose the most effective remedy. Identifying the underlying genetic variations and understanding their functional impact may enable clinicians to identify individuals at high risk of sustaining osteoporotic fractures and researchers to develop new therapeutic agents. Thus far, genome-wide association studies in large populations have failed to identify clinically significant genomic variations. Findings regarding some genes and pathways have been replicated, but in many studies the results have diverged. Hereto discovered variations have only modest effects on bone density. The few known significant genes linked to osteoporosis have been pinpointed by linkage studies of affected families, one example being the *low density lipoprotein receptor-related protein 5* (*LRP5*) (Gong *et al.*, 2001). This gene functions in the so-called Wnt pathway, which is an extensively studied but still not completely understood pathway affecting bone accrual and renewal (Westendorf *et al.*, 2004).
This study focuses on monogenic forms of osteoporosis and the role of *LRP5* in early-onset disease. Children and families exhibiting significant osteoporotic bone phenotypes were examined with the goal of identifying clinical and genetic patterns, and ultimately new genetic variations, behind this complex disease.

2. REVIEW OF THE LITERATURE

2.1 BONE HEALTH AND OSTEOPOROSIS

2.1.1 Bone health and normal structure

Bones form the supportive backbone of the body, functioning as the main storage of minerals and as the center for hematopoiesis. Bones are composed of bone tissue and of bone marrow, endosteum, periosteum, nerves, blood vessels, and cartilage. Bone tissue, a dense mineralized connective tissue, is mainly composed of calcium hydroxyapatite, which gives bones their rigidity. The elasticity of bones is provided by the organic components, mainly type I collagen fibers. This combination of hard mineral and flexible collagen makes bone hard and strong without being brittle.

There are two types of bone tissue, compact and trabecular, which have identical biology but different micro-architecture. The main part of bone mass is contained in compact bone, which is of low porosity and forms the hard, smooth bone surface. The interior is filled with trabecular bone bridges, hematopoietic bone marrow, and fatty tissue. Trabecular bone is of high porosity and since it contains 90% of the total bone surface, it constitutes the metabolically active area.

The bone-forming osteoblasts and osteocytes are mononucleate cells descending from osteoprogenitor cells of mesenchymal stem cell origin (Figure 1). They communicate with other osteocytes and osteoblasts through long cytoplasmic processes, which also function as sensory receptors for mechanical loading. Bone resorption occurs at bone surfaces by osteoclasts, which are large, multinucleated cells of monocyte stem cell origin (Figure 1). Osteoclasts resorb bone by secreting enzymes and by phagocytic mechanisms. (Coe *et al.*, 2002)

Bone remodeling is the coordinated process of bone resorption followed by new bone formation for the maintenance of calcium homeostasis and for damage repair. Bone modeling, on the other hand, is an uncoupled process of bone formation and degradation, mostly regulated by mechanical forces. Bone modeling is necessary to shape the skeleton during growth and to maintain bone shape and mass. (Giustina *et al.*, 2008) Bone is continuously built and degraded simultaneously and these two processes, together termed bone turnover, are tightly interconnected to maintain calcium homeostasis and bone mass. Systemic hormone levels affect bone turnover both through direct binding to receptors and indirectly by affecting local cytokine production in a complex manner (Table I). Bone turnover is mainly orchestrated by osteoblasts that secrete cytokines which either stimulate or inhibit osteoclast differentiation and activity. The most important regulatory pathway in bone metabolism is the

RANK/RANKL/OPG-pathway, which involves the osteoclastic receptor for receptor activator of nuclear factor-kappaB (RANK) and its ligands: RANK-ligand (RANKL) and the decoy ligand osteoprotegerin (OPG); both secreted by osteoblasts (Hofbauer *et al*., 2000). High levels of RANKL shift bone turnover towards resorption, while an increase in OPG diminishes bone loss and enables an increase in bone mass. The main activator of osteoblast activity is the canonical Wnt pathway (Glass *et al*., 2005), which is presented in detail in Chapter 2.3.

Bone formation and resorption occur continuously throughout life. Bone mass increases in childhood and adolescence, reaching a plateau level in early adulthood. Up to 90% of this peak bone mass is acquired before the age of 18, and over 25% of adult total bone mass is accrued during the 2 years around peak bone mineral density (BMD) velocity (Bailey *et al.*, 1999). After puberty, bone mass accumulation decreases rapidly (Theintz *et al.*, 1992) and the bone mass remains rather stable between ages 20 to 30. Thereafter a slow age- and hormone-related loss of bone mass can be observed, rapidly accelerating after menopause in women and 5-10 years later in men.

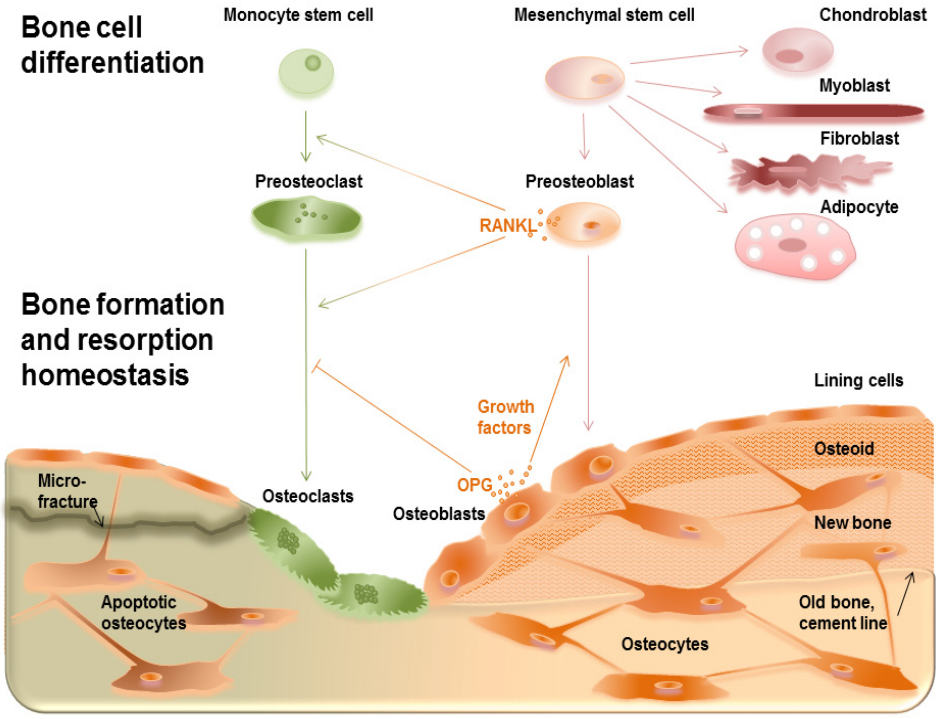


Figure 1. Bone forming cells, osteoblasts and osteocytes, are of mesenchymal stem cell origin. Osteoblasts produce the collagenous matrix, or the osteoid, and the enzyme alkaline phosphatase, which enables mineralization of the osteoid. As the mineralized matrix hardens into new bone, osteoblasts become entrapped and develop into osteocytes, which are mature bone cells responsible for matrix maintenance and calcium homeostasis. Osteoblasts and osteocytes communicate through cytoplasmic processes and control the bone turnover rate in a paracrine fashion by secreting cytokines which affect the differentiation and activity of bone osteoclasts and osteoblasts.

Table I. The hormonal regulation of bone turnover is complex and involves many interacting factors. The main hormonal regulators of bone turnover (by endocrine signaling) are presented, along with a selected number of local cytokines representing autocrine and paracrine signaling in bone.

Hormone or cytokine	Secreted by	Action	Notes	Reference
Regulators of bone formation				
Glucocorticoids	Adrenal cortex	induction of apoptosis of OBs and osteocytes	Secondary recruitment of osteoclasts occurs after apoptosis. Inhibits IGF-1 and GH function in bone.	Canalis *et al*., 2007
Growth hormone (GH)	Pituitary gland	increases osteoblastogenesis	Direct action via GH-receptor, indirect action via stimulated production of IGF-1, OPG, bone morphogenic proteins (BMPs), and PTH, via increases in phosphate and $1,25(OH)_2D3$. Production stimulated by hypothalamic gonadotropin-releasing hormone (GnRH) and progesterone.	Giustina *et al.*, 2008, Sun *et al*., 2006
Insulin-like growth factor 1 (IGF-1)	Liver (systemic action)	enhances osteocyte function, inhibits apoptosis	Direct action via receptors on OBs. Maintains cortical bone.	Giustina *et al.*, 2008
	Osteoblasts (local effect)	enhances osteocyte function, upregulates collagen production	Production upregulated by PTH, (not GH), and estrogen, thyroid hormones and BMPs. Maintains trabecular bone.	Giustina *et al.*, 2008
Insulin	Pancreatic β-cells	enhances bone formation and collagen production	Direct action via receptors on OBs.	Pun *et al.*, 1989, Fulzele *et al*., 2012
Calcitriol ($1,25(OH)_2D3$)	Formed in skin, activated in liver and kidney	increases bone formation	Induces the expression of Wnt receptor LRP5, affects PTH expression and calcium uptake.	Fretz *et al*., 2007
Runt-related transcription factor 2 (RUNX2)	Osteoblasts	increases OB differentiation	Optimal level maintained by sex hormones.	Frenkel *et al*., 2010
Wnt-ligands	Osteoblasts, + other cells?	induce proliferation of OBs and bone formation	Auto- and paracrine regulation of ligand and receptor expression patterns.	Westendorf *et al*., 2004
Osteoblastic growth factors	Osteoblasts, chondrocytes	increase bone formation	Production stimulated by PTH, GH, $1,25(OH)_2D3$.	Huang JC *et al*., 2004, Hofbauer *et al*., 1998
Parathyroid hormone (PTH)/ PTH-related peptide (PTHrP)	Parathyroid gland / osteoblasts	increases OB recruitment and inhibits OB apoptosis	Direct action via receptors on OBs. Effect seen *in vivo* when PTH is administered intermittently. PTHrP effect is in part due to enhancement of Wnt-signaling.	Qin *et al*., 2004, de Castro *et al*., 2011, Miao *et al*., 2005
Regulators of bone resorption				
Parathyroid hormone (PTH)	Parathyroid glands	stimulates bone resorption	Direct action via receptors on OCs. Also decreases the OPG/RANKL ratio from OBs when PTH is continuously administered.	Teti *et al*., 1993, Qin *et al*., 2004
Thyroid-stimulating hormone(TSH)	Pituitary gland	inhibits OC activity	Direct action via receptors.	Ma *et al*., 2011
Calcitonin (CLN)	Thyroid gland	inhibits OC activity	Direct action via receptors, production stimulated by high blood calcium levels.	Turner *et al*., 2011
Thyroid hormones (T4 and T3)	Thyroid gland	high levels cause high bone turnover and bone loss	Direct nuclear receptors (THα and THβ). Thyroid hormones also regulate IL-6 production.	Coe *et al*., 2002, Monfoulet *et al*., 2011, Roef *et al*., 2011
Follicle-stimulating hormone (FSH)	Pituitary gland	increases OC differentiation and activity	Direct action via receptors on OCs.	Sun *et al.*, 2006
Estrogen	Ovaries	inhibits OC differentation	Direct action via ERα-receptors. Increases the OPG/RANKL ratio by inhibiting cytokines (e.g. IL-6).	Hofbauer *et al.*, 1999, Sun *et al*., 2006
Androgens	Testes, adrenal cortex	both positive and negative effects on bone mass	Decreases OPG and RANKL expression in OBs, inhibits IL-6 effect. Has estrogen effects after aromatization.	Hofbauer *et al*., 2002; Huber *et al.*, 2001
Receptor activator of nuclear-κβ ligand(RANKL)	Osteoblasts	increase OC differentiation and activity	Production stimulated by PTH, $1,25(OH)_2D3$, and RUNX2. Production inhibited by sex hormones, TSH, and FSH.	Blair *et al*., 2006
Osteoprotegerin (OPG)	Osteoblasts	inhibits OC function	Production stimulated by estrogen, mechanical load, and $1,25(OH)_2D3$.	Frenkel *et al*., 2010, Yu HC *et al*., 2010, Hofbauer *et al*., 1998
Interleukin-6 (IL-6) and other cytokines (e.g. IL-1, TNFα)	T-cells, monocytes, fibroblasts	upregulates osteoclastogenesis	Increases RANKL-expression, production inhibited by estrogens and androgens.	Bellido *et al*, 1995; Le Goff *et al*., 2010

OB, osteoblast; OC, osteoclast.

2.1.2 Assessment of bone health

There are many means to assess bone health. I) Bone turnover can be estimated with blood and urine markers reflecting the bone formation and resorption rates. II) Bone mass can be measured with methods estimating areal or volumetric BMD. III) Assessment of bone quality and micro-architecture by histomorphometric studies from bone samples provides the most exact information on bone strength and turnover. In the clinical setting, assessment of bone health becomes relevant if an individual presents with recurrent fractures, or suffers from medical conditions or uses certain medications known to cause deterioration of bone health. Some of the methods mentioned below are mainly utilized in research settings.

Biomarkers of bone health

During bone formation and resorption, protein cleavage products and enzymes are released into the circulation and excreted into the urine. These can be assessed by standardized laboratory methods available at most hospitals. The markers are not valid in the diagnosis of osteoporosis, but provide additional information on the rate of bone remodeling and can be used as a complement to BMD measurements, e.g. in the follow-up of osteoporosis treatments. The most commonly used markers for **bone formation**, reflecting osteoblast activity, are all measured from serum. Deposition of the organic matrix is reflected by procollagen type I C- and N-terminal peptides (PICP, PINP). Bone-specific alkaline phosphatase (BALP) relates to the organization and calcification of the matrix, and the calcium-binding non-collagenous protein osteocalcin (OCN) is maximally expressed during mineralization of the matrix. (Caulfield and Reitz, 2004; Hauschka *et al.*, 1989; Lewiecki, 2010) **Bone resorption** can be assessed by measurement of collagen degradation products: C-terminal telopeptide of type I collagen (ICTP) from serum and N-telopeptide of type I collagen (NTX) from serum or urine. Collagen degradation also releases pyridinium cross-links, which can be measured from urine as deoxypyridinoline (DPD). Tartrate-resistant acid phosphatase isoform 5b (TRAP-5b) is an enzyme released by osteoclasts which dissolves the mineral matrix, and is measured from serum samples (Caulfield and Reitz, 2004). The reference values of these markers are age- and sex specific (Seibel, 2005, 2006).

Measurement of bone mass and mineral density

The golden standard for bone health assessment is BMD measurement by **dual-energy X-ray absorptiometry** (DXA), which is the base for the diagnosis of osteoporosis according to the guidelines of the World Health Organization (WHO, 1994). DXA is the most commonly used tool for assessing BMD due to its wide availability and low radiation dose. DXA measures bone mineral con-

tent (BMC) and bone area, and these are expressed as areal BMD (aBMD) in grams per square centimeter. The result is compared with the mean aBMD of young healthy adults, and the deviation from this mean value in standard deviation (SD) units is designated the T-score. When the measured BMD value is compared with gender-, age-, and ethnicity-matched controls, the difference in BMD expressed in SDs is called Z-score. In children and adolescents only the Z-score should be used, and a diagnosis of osteoporosis should not be set in children based on BMD measurements alone (Specker and Schoenau, 2005; Lewiecki *et al.*, 2008; Rizzoli, 2010).

Areal BMD does not fully take bone size into account. A more reliable means for assessing BMD is therefore a volumetric measurement. **Quantitative computed tomography** (QCT) from the spine or from peripheral measuring sites (from the radius, tibia or femur) provides three-dimensional data on bone mineral density, geometry, and size, as well as allows for distinction between the cortical and trabecular bone compartments. The radiation dose for spine measurements is much higher than in DXA, but peripheral measurements provide reliable results with lower radiation (Specker and Schoenau, 2005).

Quantitative ultrasound (QUS) measures the attenuation of the ultrasound beam when it passes through bone. Reference values for calcaneal and phalangeal measurements are available, but only calcaneal measurements are validated in clinical use for osteoporosis management. QUS can be used for preliminary screening as the measuring device is portable, inexpensive, and there is no exposure to radiation. (Specker and Schoenau, 2005; Lewiecki *et al*., 2008)

Assessment of bone histology

The most accurate quantitative information regarding bone micro-architecture and turnover is obtained by histomorphometric studies of bone biopsy specimens. The sample specimen should be obtained from the anterior superior iliac spine by standardized methods and should be intact and transiliac, i.e. contain two cortices separated by trabecular bone. Histomorphometric analysis is helpful for both accurate diagnosis and treatment follow-up of metabolic bone diseases. It provides exact quantitative information on the amount and distribution of bone and bone cells in the sample. For assessment of dynamic bone parameters, such as mineral apposition rate and bone formation rate, a two-course per oral tetracycline labeling is required prior to the biopsy (Rauch, 2003). The acquisition of a bone biopsy sample requires an invasive procedure and is therefore more sparingly used in the clinical setting. The biopsy sample can be scanned with micro-CT for three-dimensional fine trabecular detail analysis. Other alternatives for assessment of micro-architecture are high-resolution CT and magnetic resonance imaging (MRI), but these are used only in research settings due to cost, poor availability, and lack of consensus regarding analytic standards. (Griffith and Genant, 2008)

2.1.3 Osteoporosis

Definition, prevalence and morbidity

Osteoporosis is a metabolic skeletal disorder, in which compromised bone strength predisposes an individual to a higher risk of fragility fractures. According to the WHO osteoporosis criteria, bone fragility arises from a decrease in bone mass and from micro-architectural deterioration without disturbances in mineralization (WHO, 1994). Osteoporosis is the most common skeletal condition in the Western world; the lifetime risk of fractures associated with osteoporosis exceeds 40% in women and 20% in men (Kanis *et al.*, 2000). Approximately 10 million people in the United States are estimated to suffer from osteoporosis and 18 million more have osteopenia, or decreased BMD, yielding a prevalence of low bone mass exceeding 10% in the population (Osteoporosis prevention, 2001). The estimated annual financial expenditures were estimated to $10-$15 billion in 2001 in the US, not including preventive care nor morbidity-related lost wages and productivity (Osteoporosis prevention, 2001).

Apart from the financial consequences, osteoporosis-related fractures cause a significant decrease in the patients' quality of life; this applies to both hip and vertebral fractures (Lips *et al.*, 2005; Salaffi *et al.*, 2007; Willig *et al.*, 2001). In the majority of affected patients the condition permanently affects functional ability to carry out day-to-day chores, social activities, and interferes with family relationships (Roberto, 2004). Two thirds of patients who have sustained a hip fracture never regain their pre-fracture level of function (Willig *et al.*, 2001). Sustaining fractures is also associated with excess mortality; the one-year mortality after a hip fracture is 20% (Piirtola *et al.*, 2008).

Diagnosis

The WHO definition of osteoporosis is only applicable to BMD measurements using DXA, and the diagnostic threshold values have been set according to data from postmenopausal women. The clinical diagnosis of osteoporosis is based on the T-score, with a T-score of less than -1.0 being defined as osteopenic and a T-score of less than -2.5 as osteoporotic (WHO, 1994). In healthy children and adolescents, BMD increases continuously and varies with age, gender, height, skeletal maturation, and pubertal status. The measured BMD value is therefore compared with gender-, age-, and ethnicity-matched controls. Due to great individual variation in maturation during childhood and puberty, BMD should further be adjusted for skeletal maturity and/or height, as appropriate (Specker and Schoenau, 2005; Gordon *et al.*, 2008; Rizzoli, 2010). In young adults and children, Z-scores below -2.0 are considered "low bone mass for chronologic age" (Lewiecki *et al.*, 2008).

When examining a patient with suspected bone weakness, laboratory tests for parameters of calcium homeostasis, vitamin D levels, and specific tests to exclude causes of secondary osteoporosis, e.g. celiac disease antibodies and thyroid function markers, should be considered based on patient history and clinical findings. Bone turnover markers may be useful, e.g. in the follow-up of treatment response.

Histomorphometric studies are mainly used in research settings, but can aid the clinician in the establishment of diagnosis and in treatment follow-up. Apart from quantitative information on the amount and distribution of bone and bone cells in the sample, they provide information on mineralization and bone turnover.

Etiology

Osteoporosis can occur in both sexes and at all ages, and is etiologically divided into primary and secondary osteoporosis (Table II). The most common form of osteoporosis is age-related and strongly associated with the decrease of sex hormones in the elderly population. The most significant risk factors for osteoporosis are female sex, high age, Caucasian race, low body mass index, estrogen deficiency, family history of bone fragility, history of previous fractures, glucocorticoid therapy, and use of tobacco and alcohol (Kronenberg *et al.*, 2008; Osteoporosis prevention, 2001; Sandhu and Hampson, 2011).

Primary osteoporosis can arise due to; I) failure to achieve optimal peak bone mass in adolescence, II) excessive resorption of bone, or III) failure to adequately replace the resorbed bone, i.e. through a bone formation deficit. With increasing age and declining sex hormone levels, the bone resorption rate exceeds that of bone formation and a physiological rapid decline in BMD occurs in women after menopause and in men around the age of 60-65 years. After post-menopausal osteoporosis the most common cause of primary osteoporosis is osteogenesis imperfecta (OI) caused by mutations in the genes coding for type I collagen and related peptides (Roughley *et al.*, 2003; Rauch and Glorieux, 2004). The typical features of OI are bone fragility with multiple peripheral and vertebral compression fractures, blue sclerae, excessive joint laxity, dentinogenesis imperfecta, and hearing loss (Roughley *et al.*, 2003). Other hereditary forms are known (summarized under Genetics of osteoporosis; Table III), but they are rare. There is strong evidence for significant heredity in the remaining patients, but as the genetic background is unknown and some patients have no family background of bone disease, these cases are termed idiopathic.

Secondary osteoporosis arises as a complication of many medical conditions and drugs, which can affect bone strength and predispose patients to fragility fractures (Table II). The most common form of secondary osteoporosis is connected to the long-term use of glucocorticoids, which causes a significant

decrease in BMD even at low dosage (Laan *et al.*, 1993). Other drugs affecting bone metabolism and strength include, for example, anticonvulsants, chemotherapeutic agents, heparin, glitazones, and gonadotropin-releasing hormone analogues (Kaufman *et al*., 2008; Kiratli *et al.*, 2001). Osteoporosis can be a feature of certain genetic and hereditary disorders as well as many endocrine, nutritional or gastrointestinal, hematopoeitic, connective tissue, and severe chronic disorders. (Kaufman *et al.*, 2008; Kronenberg *et al.*, 2008).

Adolescence is the crucial time for bone mass accrual. After the age of 20 years, only approximately 5-10% of peak bone mass is acquired. Regarding peak bone mass, the strongest predictor is heredity (Bonjour *et al.*, 2007), but it is also affected by factors such as nutrition, physical activity, and illnesses (Bailey *et al.*, 1999; Stewart & Ralston 2000). Even if these non-genetic factors were transient, they can affect bone health in later life (Rizzoli *et al.,* 2010). Conversely, by ensuring adequate calcium and vitamin D intake in children, a higher peak bone mass can be achieved with possible benefits in adulthood (Bonjour *et al.*, 2001). In many patients, bone weakness may become symptomatic in late adulthood despite the fact that their bone structure may have been suboptimal since adolescence. Based on a computer simulation, the onset of osteoporosis in adulthood may be postponed by 13 years if the individual has achieved a 10% higher peak bone mass than the average (Hernandez *et al.*, 2003). This shows that the optimal time to intervene for the prevention of osteoporosis is, in fact, during adolescence.

Table II. Causes of osteoporosis.

Primary

Aging
Genetic / hereditary
Idiopathic

Secondary

Endocrine disorders
- Acromegaly
- Cushing's syndrome
- Diabetes mellitus (type 1)
- Hyperparathyroidism
- Hyperthyroidism
- Hypogonadism

Nutritional, gastrointestinal and malabsorption disorders
- Chronic pancreatitis
- Cystic fibrosis
- Gastrectomia, bariatric surgery
- Hepatobiliary disease
- Inflammatory bowel disease
- Malabsorption syndromes (e.g. coeliac disease)
- Pernicious anaemia
- Total parenteral nutrition
- Vitamin and mineral deficiency

Connective tissue disorders
- (Osteogenesis Imperfecta, often included in primary osteoporosis)
- Ehlers-Danlos syndrome
- Homocystinuria
- Marfan's syndrome

Hematopoietic disorders
- Haemochromatosis
- Leukemias, lymphomas
- Mastosytosis
- Myeloproliferative disorders
- Plasma cell dyscrasias

Others
- Alcoholism
- Chronic obstructive pulmonary disease
- End-stage renal insufficiency
- Hypercalciuria
- Hyperhomocysteinaemia
- Immobilization
- Medications
- Neoplastic diseases
- Post-transplantation
- Rheumatoid arthritis

2.2 GENETICS

2.2.1 The human genome and its variation

Normal structure of genes

The human genome consists of 22 pairs of autosomal chromosomes and one pair of sex chromosomes; XX for females and XY for males. All cells contain the identical genetic code inherited from the parents. The chromosomes contain the genetic code as densely packed deoxyribonucleic acid (DNA) in helical coils built up from a chain of four bases: adenosine (A), thymine (T), guanine (G), and cytosine (C). These bases form pairs; A-T and G-C, and therefore DNA is double-stranded. Human DNA contains approximately 3 billion base pairs, in addition to a small amount of maternally inherited mitochondrial DNA.

The genetic code is divided into gene-containing coding regions or exons, and non-coding regions or introns. Exons contain the information for protein synthesis and introns can contain regulators of gene transcription such as promoters, response elements, enhancers, and silencers.

For intracellular production of proteins, the DNA is first copied, transcribed, in the nucleus into single-stranded messenger ribonucleic acid (mRNA), which after exiting the nucleus binds to small organelles in the cytoplasm called ribosomes. Here the mRNA is spliced in such a way that sections unnecessary for the production of proteins, the introns, are spliced off and translation into proteins occurs only from the genomic code contained by the exons. In the translation from mRNA to proteins, one triplet of bases encodes for an amino acid most frequently starting from the amino acid methionine (Met), which functions as the start codon, and ending in a stop codon.

After translation, the protein can be cleaved, modified by addition of e.g. lipids, sugar chains, or phosphate groups, and it coils into a three-dimensional structure, which often is crucial for the correct function of the protein. Proteins have multiple functions and can serve as building material, hormones, enzymes, receptors, and transcription factors in the body. (Strachan *et al*., 1996)

Genetic variation

The most common form of genetic variation is a single-base variation, or single nucleotide polymorphism (SNP), which occurs in approximately 1:1000 bases (Ng *et al*., 2008). Less than 1% of SNPs are thought to be of functional significance. A SNP which does not result in the alteration of an amino acid is called synonymous, and if the encoded amino acid changes, the variation is called non-synonymous. If the non-synonymous variation alters the function of the

protein, it is called a missense mutation. In a nonsense mutation the change of nucleotide introduces a premature stop codon resulting in premature termination of translation.

The additions or removal of nucleotides from the genetic sequence are called insertions or deletions. Due to the three-base reading frame in protein translation, a single insertion or deletion can change the reading frame of the coding sequence (a so-called frameshift mutation) resulting in the complete alteration of the subsequent protein or in the introduction of a premature stop codon.

SNPs, insertions, and deletions can also change the way in which the mRNA is spliced at the ribosomes, especially if the genomic variation occurs at the exonic or intronic bases adjacent to the splice site at the beginning, or at the end, of an exon. These changes are called splice site mutations and can cause the exclusion of a complete exon, the activation of secondary splice sites, and the change of the reading frame of the sequence.

Genetic variation also includes repetitions of parts of the sequence, sometimes multiple repetitions of only two or three nucleotides (so-called microsatellites) to large duplications. Chromosomal changes also include inversions (the rotation of a sequence segment) and translocations (the exchange of sequence segments between chromosomes). The impact of the genetic variation varies vastly with the site it affects: a mutation caused by the change of a single base can cause a lethal phenotype, while large deletions can remain asymptomatic (Strachan *et al*., 1996; Brown, 2003). The majority of genetic variations in humans are functionally neutral (Ng *et al*., 2008). Missense mutations, and even nonsense and frameshift mutations, in functional genes may be of no clinical relevance due to multiple gene copies and gene redundancy based on evolution (Ng *et al*., 2008).

2.2.2 Genetic screening and mapping

When looking for disease-causing genetic changes, the mode of inheritance must be taken into account. According to Mendelian laws, a dominant disease is inherited as a single defective allele from one parent, whereas a recessive disease requires the inheritance of a faulty allele from both parents. As the presumption is that Mendelian inheritance is caused by a single causative gene or variation, this mode of inheritance is called simple. In complex inheritance there are multiple loci affecting the phenotype, each often with a small effect. Diseases of Mendelian inheritance are often rare, possibly due to poorer reproductive fitness. Common diseases, such as type-2 diabetes, are often due to complex inheritance with a multitude of genetic variations rendering the individual susceptible to or protected from the disease.

Traditionally, the heredity of disorders has been investigated through **twin studies**. Since environmental factors are shared among twins, and monozygotic twins share 100% and dizygotic twins 50% of their genotype, the environmental effect vs. heritability for a certain phenotype can be assessed.

Due to the different modes of inheritance, different approaches to identify the underlying genetic changes are necessary. In diseases with Mendelian inheritance, pedigree studies with **linkage analysis** are useful. The genome is screened using markers, e.g. SNPs or microsatellite repeats. As nearby markers are often inherited together in "blocks", a marker inheritance pattern can be created revealing a possible linkage between disease phenotypes and certain genetic markers. For statistical analysis, a logarithmic odds score (LOD score) is calculated, which is equal to the logarithm to base 10 of the ratio of the "probability of linkage" to the "probability of non-linkage" of the marker with respect to the investigated phenotype. A LOD score of ≥ 3 (which corresponds to the odds of 1000:1) is generally regarded as acceptable evidence for linkage. Linkage analysis on the association of the phenotype to single microsatellite markers is done by two-point LOD-score analysis, whereas a multi-point LOD-score analysis takes into account whether multiple adjacent markers link to the investigated phenotype. The strength of linkage analysis is that monogenic inherited diseases can be found based on a limited number of affected individuals, provided that they are related. The draw-back is that after the identification of linkage regions, pin-pointing the underlying gene and its mutation can demand time-consuming sequencing of large genetic regions.

If the individuals are not related, the investigated phenotype can be caused by sequence variations in different genes in the genome, which renders the use of linkage analysis impossible. The approach taken is then "case vs. control", where healthy controls should be genetically matched to the affected patients (i.e. chosen from the same population). Markers in candidate genes for a particular disease in **candidate gene association studies** or genome-wide

markers (common SNPs) in **genome-wide association studies** (GWAS) are analyzed. The frequency of these genetic markers is compared between cases and controls and if a difference in frequency between these groups is discovered, the marker is considered to be associated with the phenotype. The weakness of the candidate gene approach is that only selected parts of the genome are screened, and the valid selection of candidate genes can be demanding. As the cost of genome-wide studies has become manageable, the genome-wide approach has gained popularity. The draw-back of association studies, however, is that large numbers of both cases and controls are required to achieve sufficient statistical power. (Strachan *et al*., 1996; Brown, 2003)

Recently, the combination of **next-generation sequencing**, where large areas of the genome can be sequenced in one run, and the so-called **target enrichment**, where specific regions of the genome are selected and enriched for sequencing, has enabled researchers to directly sequence e.g. large linkage areas or even all exons in the genome in a single run (Hodges *et al*., 2007). This new method will aid in identifying rare disease-causing genetic changes and, as the cost for the method falls, also by-pass the need for linkage and association studies (Ng *et al.*, 2009). The difficulty with this approach is filtering the results. Based on the results from a whole-exome sequencing project, one healthy adult carried approximately 12500 coding variants which alter protein sequence. Of the over 10000 non-synonymous SNPs 7% were novel and over 100 induced a premature stop codon. The screened individual carried over 700 insertions or deletions in coding areas, of which half caused a frameshift of the reading frame. Singling out a disease-causing mutation from this amount of variations may prove to be an arduous task. (Ng *et al*., 2008)

2.2.3 Genetics of osteoporosis

Susceptibility genes for osteoporosis

Osteoporosis is a highly inherited trait; twin and family studies have revealed the heredity among first-degree relatives to be from 50% to 80% (Howard *et al.*, 1998; Stewart & Ralston 2000, Ralston et Crombrugghe 2006). The heredity of osteoporosis has traditionally been considered to be complex, with many predisposing factors together causing the phenotype. In segregation analyses of pedigrees with extreme BMD values, an estimated 16% of the variation of BMD is explained by monogenic traits and an additional 56% by residual polygenic effects. No evidence has been found to support an underlying aetiology of shared familial environmental effects in these studies (Spotila *et al.*, 1996; Cardon *et al.*, 2000, Deng *et al.,* 2002). There are a few reports on dominantly inherited osteoporosis with high penetrance, but despite significant and small linkage regions, the causative genes are yet to be identified in these pedigrees (Vidal *et al.*, 2007; Willaert *et al.*, 2008) with the exception of OI-causing muta-

tions, some rare syndromes, and mutations in the genes encoding low density lipoprotein receptor-related protein (*LRP*) 5 and *LRP6* (Gong *et al.*, 2001; Mani *et al.*, 2007). (Table III)

Interestingly, the underlying genetic predispositions for low BMD and increased fracture risk seem to diverge. A positive fracture history in family members correlates with an individual's risk for fragility fractures and micro-architectural deterioration in bone, but not always with the risk of low BMD (Deng *et al.*; Andrew *et al.*, 2005; Genant *et al.*, 2007; Sirola *et al.*, 2008). Low BMD itself is a significant risk factor for fragility fractures. The fracture risk in women increases 1.5- to 2.6-fold with a one SD decrease in BMD (Marshall *et al.*, 1996) and in prepubertal children every one SD decrease in volumetric BMD (vBMD) increases the risk of fracture by 89% (Clark *et al.*, 2006). Nevertheless, a BMD T-score below -2.5 explains only 10% to 44% of fragility fractures (Stone *et al.*, 2003). The genes predisposing to fractures are thus unlikely to play a direct etiological role in the development of low BMD in a large scale. This divergence renders the genetic research of osteoporosis more challenging as the phenotypes overlap.

Table III. Known forms of genetic/hereditary disorders with decreased bone mineral density, excluding bone fragility from bone mineralization defects and osteopetrosis.

Name of disorder	Inheritance	MIM no.	Locus	Gene	Protein	Clinical features and notes
OI type I	AD	166200 166240	17q21-22 7q22.1	COL1A1 COL1A2	Collagen 1, alpha-1 chain Collagen 1, alpha-2 chain	mild OI, blue sclerae, IA: with DGI, IB: without DGI
OI type IIA	AD	166210	17q21-22 7q22.1	COL1A1 COL1A2	Collagen 1, alpha-1 chain Collagen 1, alpha-2 chain	perinatally fatal
OI type IIB	AR	610854	3p22	CRTAP	Cartilage-associated protein	perinatally fatal, see also OI type VII
OI type III	AD AD/AR	259420	17q21-22 7q22.1	COL1A1 COL1A2	Collagen 1, alpha-1 chain Collagen 1, alpha-2 chain	severe, progressively deforming, normal sclerae usually AD, rarely recessive COL1A2 mutations causing severe OI
OI type IV	AD	166220	17q21-22 7q22.1	COL1A1 COL1A2	Collagen 1, alpha-1 chain Collagen 1, alpha-2 chain	moderate OI, normal or grey sclerae, often DGI
OI type V	AD	610967				moderately deforming, normal sclerae, no DGI. Hyperplastic callus, calcificied interosseous membranes, radioopaque metaphyseal bands, irregular mesh-like lamellae on biopsy
OI type VI	AR	610968	17q21	FKBP10	FK506-binding protein 10	faintly blue sclerae, no DGI or wormian bones, bone histology with fish scale-like lamellae and excessive osteoid
OI type VII	AR	610682	3p22	CRTAP	Cartilage-associated protein	OI with rhizomelia, see also OI type IIB
OI type VIII	AR	610915	3p22-p24.1	LEPRE1	Leucine- and proline-enriched proteoglycan 1	white sclerae, severe growth deficiency, extreme skeletal undermineralization, and bulbous metaphyses
OI type IX	AR	259440	15q21-q22	PPIB	Peptidyl-prolyl isomerase B	clinical features of moderate to severe OI, compatible with OI IIB / III
OI type X	AR	613848	11p13.5	SERPINH2	Serine proteinase inhibitor	Severe OI, DGI, blue sclerae
OI type XI	AR	613849	12q13.13	SP7	Osterix	Moderate to severe OI, normal sclerae and hearing, no DGI, delayed tooth eruption
OI type XII	AR	613982	17p13.3	SERPINF1	Pigment epithelium-derived factor (PEDF)	severe OI, grey sclerae, no DGI or hearing loss
Bruck syndrome 1	AR	259450	17p12?	FKBP10?	FK506-binding protein 10	OI with pterygia
Bruck syndrome 2	AR	609220	3q23-24	PLOD2	Procollagen lysyl hydroxylase 2	OI with pterygia
OPPG	AR	259770	11q12-13	LRP5	LDL-receptor related protein 5	severe osteoporosis, early-onset blindness
BMD quantitative trait locus 1	AD	166710	11q12-13	LRP5	LDL-receptor related protein 5	decreased bone mineral density
ADCAD2	AD	610947	12p13.2	LRP6	LDL-receptor related protein 6	early-onset coronary disease and primary osteoporosis
Singleton-Merten dysplasia	AD	182250				widened medullary cavities of bone, aortic calcification, abnormal dentition, and muscular weakness
Geroderma osteodysplasticum	AR	231070	1q24.2	SCYL1BP1	SCYL1-binding protein 1	wrinkly skin and osteoporosis
Calvarial doughnut lesions, bone fragility	AD	126550				pathologic fractures, lumps on the head, elevated serum alkaline phosphatase levels, and dental caries
Idiopathic juvenile osteoporosis	unknown	259750				heterogenous group of osteoporotic children, some with bone pain but without other distinct clinical features
Cole-Carpenter dysplasia	unknown	112240				bone fragility with craniosynostosis, ocular proptosis, hydrocephalus, distinctive facial features
Spondylo-ocular syndrome	AR	605822				moderate osteoporosis, platyspondyly, advanced bone age, cataract, retinal detachment, facial dysmorphism, short trunk, immobile spine, kyphosis.
Cleidocranial dysplasia	AD	119600	6p21	RUNX2	Runt-related transcription factor 2	persistent fontanels, hypoplasia/aplasia of clavicles, wide pubic symphysis, dental and digital anomalies, in some cases severe osteoporosis, scoliosis
Gnathodiaphyseal dysplasia	AD	166260	11p14.3	ANO5	Anoctamin 5	frequent fractures in adolescence, purulent osteomyelitis of the jaws in adulthood
Hadju-Cheney syndrome	AD	102500	1p13-p11	NOTCH2	Notch2	Osteoporosis, facial abnormalities, acro-osteolysis, hearing loss, renal cysts

OI: osteogenesis imperfecta; OPPG: osteoporosis-pseudoglioma syndrome; DGI: dentinogenesis imperfecta; AD: autosomal dominant; AR: autosomal recessive. Adapted and updated from Superti-Furga et al [2007].

Results from linkage and association studies

Numerous genome-wide linkage and association studies have been performed aiming at identifying loci that regulate BMD. The results from the most relevant studies are summarized in Table IV (with data on the corresponding publications in Table V). Only few of the loci meet the criteria set for genome-wide significance.

Taking into account the large number of studies performed, a surprisingly low replication rate of susceptibility loci can be found. This may signify that the loci in different ethnic populations differ from each other. Many of the genes seem to regulate bone strength in a site- and gender-specific manner, and the genes that regulate peak bone mass acquisition differ from those affecting BMD in the elderly. There is also some evidence for a relation between osteoporosis susceptibility and parental imprinting of gene function, i.e. that the extent of gene expression depends upon the sex of the parent that transmits the gene (Duncan *et al.*, 2003). Another possible explanation for the discrepant results is that many genetic variations with modest effects exist and that the variations interact, thereby making the statistical strength of genome-wide scans insufficient for detection.

The most significant findings include variations in the genes encoding type I collagen (*COL1A1* and *COL1A2*) and in genes of specific pathways regulating bone turnover. These pathways include the Wnt pathway regulating bone formation rate (described in more detail below) and the RANK-RANKL-signaling system regulating bone cell differentiation and bone resorption involving RANK, it's ligand RANKL, and OPG; the decoy receptor of RANKL . In addition, receptors and enzymes related to estrogen and vitamin D affect bone strength in the general population. One pathway-based association study has also identified the regulation-of-autophagy (ROA)-pathway as significant in bone remodeling (Zhang *et al.*, 2010). Regulators of osteoblast differentiation also link to BMD; e.g. bone morphogenetic protein 2 (BMP2) (Styrkarsdottir *et al.*, 2003) and osterix (SP7) in the only association study performed on pediatric subjects (Timpson *et al.*, 2009).

Table IV. Genes linked to decreased bone mineral density based on association and linkage studies. *Corresponding references are marked in Table V.

Locus	Genes, according to function or pathway							Bone density phenotypes*			
	Wnt-pathway	TNF/ RANK	Estrogen -related	Calcium regulation	Vitamin D-related	ROA pathway	Other	WB/comb	LS BMD	Hip BMD	UR
1p13.3-q23.2									*V*		
1p32.2						PRKAA2					D
1p34							CSF3R		B(f)	B(f)	
1p36	ZBTB40/Wnt4	TNFR2					BGLAP	**O, P**	I, J	J, **T**	A
1q22										H	
1q23										**N**	
1q32-q43	Wnt3a							**S**(p)	*Q, V*		
2p23-24									**T**		
2q11							IL1R2		B(f)		
2q24.3										**R**	
2q35			CYP27A1				IGFBP2		B(f), L(m)		
3p25.3-p25.2						ATG7			*V*		D
3q13-q21				CASR					B(f)		
4q22							BMPR1B		L(m)		
4q22							IBSP		B(f)		
4q22							DMP1			L(m)	
4q32-34									**T**	**T**	
5p13							LIFR		B(f)		
5p15.2								**O**		*V*	
5q21-q22	APC					ATG12		**R**	L(m)	L(m), *V*	D
5q31-33										**Q**	
6p21						ATG5	MHC		J		D
6q25			ESR1						J, K	J, M	
6q26-27											**N**
7p14								**S**			
7p15							HOXA		L(m)	L(m)	
7q21				CALCR					B(f)	B(f), **T, R**	
7q33							PTN			L(m)	
8p11.21	SFRP1									E(f)	
8q24		TNFRSF11 B							C, J	C, J	
9q21.32-q31.1						IFNA		**S**(f)		*V*	D
9q31.1-q33.3										*V*	
9q34								**S**(p)			
10q24			CYP17						A	A	
10q26.13							FGFR2			L(m)	
11p11	LRP4										
11p15.2				CALCA	CYP2R1					B(f)	
11q12-13	LRP5								A, G, **Q,** *V*	E(f), G, K,M(f)	
11q23.3										**T**	
11q25								**S**			
12p13	LRP6									E(f)	
12q13	LRP1				VDR	GABARA PL1	SP7	F	H, M	E(f)	D
12q23-24									**Q**		
12q24.31-qter									*V*		
13q12-14		RANKL					FLT1		J	A, J, L(m), **Q**	
13q21.1									**R**		
14q13.1-q24.1			ESR2					**S**(f)	K	K, *V*	
14q23.3-32.12								**S**		*V*	
15q21.2			CYP19							A	A
16p12							IL4R		B(f)	B(f)	
16q24.1							FOXC2		L(m)		
17p12-q21.33	SOST					BECN1			L(m), **Q**	E(f), *V*, T	D
18pter-q12.3						PIK3C3			*V*		D
18q21-23		RANK							A	A, J, **T**	A
18q21-24		NFATC1							L(m)		
19q13					DBP		TGFB1		A	B(f)	
20p12.3							BMP2	**U**			A
21q22									**Q**		
22q11-q12							CSF2RB		B(f), **Q**(f)	B(f)	

Normal font: GWAS and SNP-based candidate gene studies, Bolded: linkage study, *Italic*: meta-analysis. WB/comb: Whole body bone mineral density (BMD) or combined BMD with lumbar spine (LS) and hip BMD or fractures, UR: ultradistal radius, (f): only in female, (m): only in male, (p): paternal imprinting.

Table V. References to Table IV. A selection of genetic studies on bone mineral density.

	Author	Year	Study setup	Country	Subject origin	Subjects (n)	Notes
A	Xiong et al.	2006	candidate gene SNPs	USA	European ancestry	1873	
B	Giroux et al.	2010	candidate gene SNPs	Canada	European ancestry	709	replication in 673 subjects
C	Richards et al.	2008	GWAS		European ancestry	2094	
D	Zhang et al.	2010	GWAS	USA	European ancestry	984	replication in 2187 subjects
E	Sims et al.	2008	Wnt candidate gene SNPs	Australia	European ancestry	344	female postmenopausal
F	Timpson et al.	2009	GWAS	USA	European ancestry	1518	only children
G	van Meurs et al.	2008	SNPs in LRP5 and LRP6		European ancestry	37543	
H	Deng et al.	2002	SNPs in VDR, BGLAP, and PTH			630	53 pedigrees
I	Spotila et al.	2000	SNPs in TNFR2				
J	Styrkarsdottir et al.	2008	GWAS	Iceland	European ancestry	5861	replication in 7924 Icelandic, Danish and Australian subjects
K	Lau et al.	2006	SNPs in selected genes	China	Chinese ancestry	674	177 pedigrees
L	Yerges et al.	2009	SNPs in selected genes	USA	European ancestry	862	QCT-based, only male, replication in 1165 subjects
M	Kiel et al.	2007	GWAS	USA	European ancestry	1141	family-based (Framingham study population)
N	Shaffer at al.	2009	GWMS	USA	Mexican American	327	32 pedigrees
O	Karasik et al.	2002	GWMS	USA	European ancestry	1270	324 pedigrees (Framingham study population)
P	Willaert et al.	2008	GWMS		European ancestry	34	one pedigree
Q	Kaufman et al.	2008	GWMS	France	European ancestry	665	103 pedigrees
R	Hsu et al.	2007	GWMS	China	Chinese ancestry	3093	siblings, from 941 pedigrees
S	Xiong et al.	2007	GWMS	USA	European ancestry	4126	451 pedigrees, combined phenotype (BMD+fractures)
T	Devoto et al	1998	GWMS	USA	European ancestry	149	7 pedigrees + sibship analysis
U	Styrkarsdottir et al.	2003	GWMS	Iceland	European ancestry	1323+478	Pedigree study, replication in 350 Danish subjects
V	Ioannidis et al.	2007	GWAS meta-analysis	mixed	European ancestry	11842	analysis in 30 cM bins

SNP: single nucleotide polymorphism; GWAS: genome-wide association study; GWMS: genome-wide microsatellite study.

2.3 THE WNT PATHWAY

The wingless-type (Wnt) pathway is a complex signaling pathway which regulates multiple developmental events both during embryogenesis and in adult tissue homeostasis. The name is derived from the first *Wnt*-genes discovered: *Wingless (Wg)* in *Drosophila* and *Int-1* in mice. Bone researchers were led to this pathway in 2001 when a co-receptor, the gene encoding *LRP5*, was identified as the causative gene behind both low and high bone mass phenotypes (Gong *et al.*, 2001; Boyden *et al.*, 2002). The canonical Wnt pathway regulates the proliferation of osteoblasts and promotes osteogenesis (Novak and Dedhar, 1999; Liu *et al*, 2009).

Wnt signaling

Signal transduction by the Wnt family of ligands is mediated by the binding to the extracellular domain receptors, including Frizzled (Fzd) and LRP. Depending on which Fzd receptor is activated the Wnt-Fzd-complex can induce activation of several signalling cascades (van Amerongen *et al.*, 2008). In the canonical pathway, the activation of the receptor complex induces the de-phosphorylation and thereby stabilization of cytoplasmic β-catenin (Figure 2). This is mediated through the activation of dishevelled (Dvl) proteins and the inhibition of glycogen synthase kinase (GSK)-3. The cytoplasmic pool of β-catenin is increased and enables this transcription regulator to enter the nucleus and promote target gene transcription by binding to responsive elements of the lymphoid enhancer factor-1/T-cell factor (LEF-1/TCF) family (Westendorf *et al.*, 2004; Liu *et al.*, 2009).

LRP5 and LRP6 co-receptors

LRP5 and LRP6 are cell membrane receptors, bound to the membrane with a single trans-membrane domain. They carry extracellular binding sites for Wnt proteins and for dickkopf 1 (DDK1), an inhibitor of Wnt-signaling (Westendorf *et al.*, 2004). The intracellular domains become phosphorylated upon forming of the LRP5-LRP6-Fzd receptor complex and thereby activate the cascade which stabilizes cytoplasmic β-catenin (Westendorf *et al.*, 2004).

LRP5 was first cloned and sequenced in 1998 (Hey *et al.*, 1998) and a few years later mutations in this gene were revealed as the cause of both low- and high BMD phenotypes, resulting from loss- and gain-of-function mutations respectively (Gong *et al.*, 2001; Boyden *et al.*, 2002; Little *et al.*, 2002). Since then, approximately 60 loss-of-function mutations in *LRP5* have been published. Homozygotes for loss-of-function mutations in *LRP5* suffer from osteoporosis-pseudoglioma syndrome (OPPG) exhibiting early-onset severe osteoporosis and blindness, and in some patients, mental retardation (Gong *et al.*, 2001).

Heterozygotes have a low BMD phenotype without eye features, and variations in *LRP5* in the general population also affect BMD (Ai *et al.*, 2005; Hartikka *et al.*, 2005; Richards *et al.*, 2008; Saarinen *et al.*, 2007, 2010; van Meurs *et al.*, 2008).

LRP6 was also first sequenced in 1998 (Brown *et al.*, 1998), but it was not until 2007 that a mutation in *LRP6* was shown to affect bone mass accrual. Only one mutation in a single pedigree has been published thus far (Mani *et al.*, 2007). The phenotypic features include early coronary disease and osteoporosis. The bone phenotype has since been replicated in a mouse model (Kubota *et al.*, 2008). Results from association studies regarding the impact of variations in *LRP6* on bone health are contradictory (Sims *et al.*, 2008; van Meurs *et al.*, 2006, 2008).

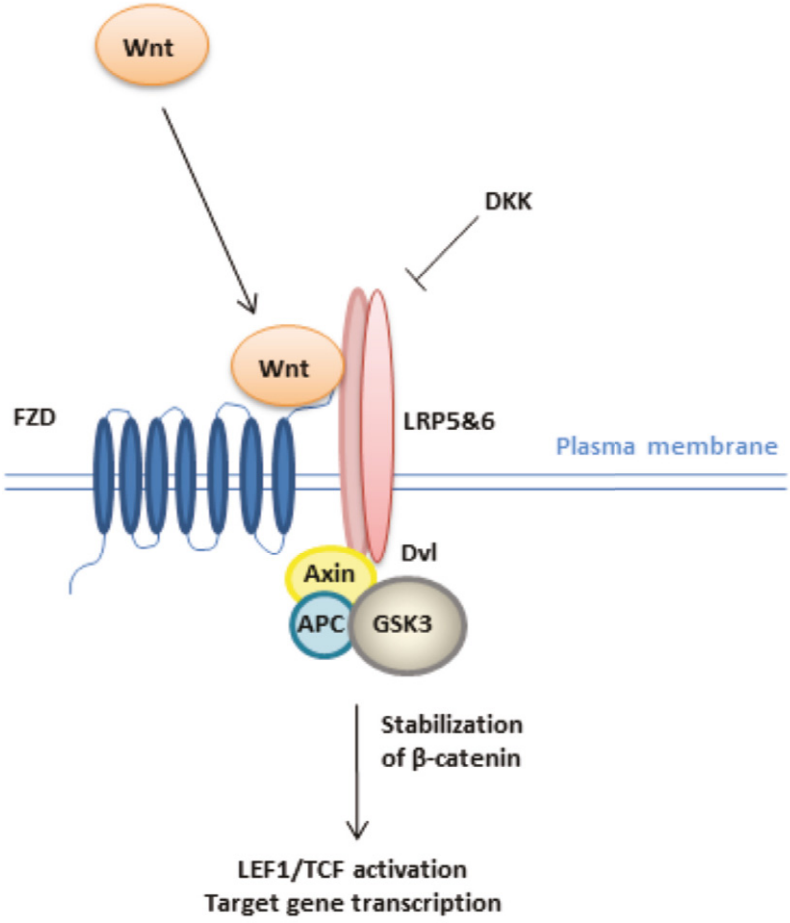


Figure 2. Schematic presentation of the canonical Wnt pathway. Wnt ligands can by binding to different receptors activate several cellular pathways, which regulate both organogenesis in embryos and tissue homeostasis in adults. In bone tissue the canonical Wnt pathway regulates osteoblast proliferation and promotes osteogenesis.

3. AIMS OF THE STUDY

Osteoporosis is a complex disease, both clinically and genetically. Despite its high frequency in the population, and extensive research in the field, only few significant osteoporosis genes have been identified to date. In this work that originated from the clinical setting, children and young adults with idiopathic bone fragility were examined and their family members were assessed in order to study the extent and mode of inheritance.

The aims of the study were:

To identify novel mutations in *LRP5* in patients with OPPG

To study the role of *LRP5* and *LRP6* in childhood onset primary osteoporosis

To identify and characterize new hereditary forms of primary osteoporosis

To discover new genes involved in the development of primary osteoporosis

4. MATERIAL AND METHODS

4.1 Consent and ethics committee permissions

Written informed consent was obtained from all participants and/or the parents, including the controls. The studies were performed with the permission of the Research Ethics Committees of Helsinki University Hospital, Helsinki, Finland (Studies I-IV); Hospital for Sick Children, Toronto, Canada (Studies II-III); and University Hospital of Cologne, Germany (Study II).

4.2 Study design, subjects and clinical assessment

Study I

A Finnish three-generation family was clinically assessed based on the high prevalence of severe osteoporosis in the pedigree. Nineteen family members consented to participate in the study at the Children's Hospital in Helsinki, Finland. All individuals underwent a thorough clinical examination, DXA (Hologic Discovery A), a radiologic assessment, and biochemical analyses on blood and urine samples (Table VI). Causes for secondary osteoporosis were excluded, if considered necessary based on clinical history, with appropriate laboratory tests, such as markers for thyroid function, celiac disease and inflammatory bowel disease. DNA from five unaffected parents of affected children was available. Four affected patients underwent bone biopsy.

An individual was considered affected if there were signs of vertebral compression fractures on spinal radiography and/or if the lumbar or femoral BMD Z-score was below -2.0 in premenopausal individuals under the age of 50 or the BMD T-score was below -2.5 in elderly or postmenopausal subjects (Lewiecki *et al.*, 2008).

As the inheritance seemed of dominant autosomal mode, genetic studies were undertaken. The linkage areas were identified by microsatellite screening of DNA samples followed by target enrichment of all exons in the linkage areas. DNA samples from 80 healthy Finns were available as controls.

Study II

Study II involves patients with a clinical diagnosis of OPPG. DNA samples from nine OPPG-patients diagnosed at clinical centers in Germany, Norway, Canada, Turkey, and Iran were screened for mutations in *LRP5.* The four patients found to carry mutations affecting protein splicing were selected for this study. Clinical data was collected from hospital records. The impact of the mutations was investigated through exon trapping, cDNA sequencing and, for one muta-

tion, by functional studies. DNA samples from 171 Finnish, 96 Caucasian (Centre d'Etude du Polymorphisme Humain; CEPH), and 50 Turkish healthy individuals served as controls.

Study III

The study includes 27 children with primary non-OI osteoporosis, diagnosed and followed at The Hospital for Sick Children, Toronto, Canada, between January 2002 and February 2008. The inclusion criteria were I) low BMD (Z score < -2.0), II) a history of three or more peripheral fractures by low-impact trauma, and/or III) vertebral compression fractures. OI was excluded clinically in 12 patients and by Sanger sequencing of *COL1A1* and *COL1A2* in 15 patients. Secondary osteoporosis was excluded with appropriate laboratory tests, e.g. tests for thyroid function, malabsorption, and sex and growth hormone levels. Clinical data was collected from hospital records, vertebral morphology was assessed by spinal radiography, and BMD was measured with DXA (Lunar Prodigy, GE Lunar, Madison, WI, USA). DNA samples from the patients were screened for variations in *LRP5, LRP6,* and parathyroid hormone-like hormone *(PTHLH)*.

Sixty nuclear family members of these patients also participated in this study by undergoing DXA of the lumbar spine. In participating adults under the age of 50 years or prior to menopause, a BMD Z-score below -2.0 was considered "below the expected range for age" (Lewiecki *et al.*, 2008). The standard definition of osteoporosis (T-score ≤-2.5) and osteopenia (T-score between -2.5 and -1.0) was used for adults over the age of 50 years and for postmenopausal women, according to international guidelines. (WHO, 1994) Control DNA samples from healthy Caucasians were screened: 171 controls for *LRP5* and 50 controls for *LRP6* and *PTHLH.*

Study IV

As a part of an ongoing project assessing clinical and genetic aspects of childhood onset primary osteoporosis, a child with primary osteoporosis was diagnosed with Calvarial doughnut lesions (CDL) at Children's Hospital in Helsinki, Finland. The proband and her father were assessed for bone health by radiography, bone densitometry (DXA, Hologic Discovery A, Hologic Inc., Waltham, MA, USA), and blood biochemistry (Table VI). Existing clinical, radiological, and biochemical data was reviewed for affected family members. Patient DNA samples were screened for mutations in the *LRP5, COL1A1*, and *COL1A2* genes. A literature review was based on previously published cases of CDL in PubMed and ISI Web of Knowledge.

4.3 Bone biopsy (Studies I and IV)

Transiliac bone biopsies were obtained under local anesthesia (adults) or under general anesthesia (child) from the anterior superior iliac crest with a bone biopsy needle of 7.5mm inner diameter (Rochester Bone Biopsy, Medical Innovations Incorporation, Inc., Rochester, MN) following a double-labeling course with oral tetracycline according to recommendations (Rauch, 2003). The bone specimens were intact and included both cortices separated by a trabecular compartment. After fixation, bone histomorphometric analyses were conducted at the Bone and Cartilage Research Unit, University of Kuopio, Kuopio, Finland, with a semiautomatic image analyzer (Bioquant Osteo, Bioquant Image Analysis Corporation, Nashville, TN). The results were compared with normative data (Glorieux *et al.*, 2000; Recker *et al*., 1988; Rehman *et al*., 1994) and presented according to the nomenclature recommended by the American Society for Bone and Mineral Research. (Parfitt *et al*., 1987)

4.4 Statistics

In study II, a two-sided Student's t-test was used to compare the signaling activity of transfected constructs. In Study III the X^2 test was applied to compare DNA variant frequencies between patients and controls. A p-value <0.05 was considered significant. The statistical software utilized in Study I for analysis of linkage LOD scores is specified below (under "Microsatellite studies").

4.5 Genetic studies

The reagents, appliances and software used in genetic studies are summarized in Table VII.

DNA/RNA purification and sequencing (Studies I-IV)

Genomic DNA was extracted from peripheral blood lymphocytes by standard methods and primers were designed to amplify exons and at least 10 bases of flanking introns (Studies I-IV). RNA was extracted from peripheral blood or cultured fibroblasts before reverse transcription polymerase chain reaction (RT-PCR) (Study II). Genomic sequences for primer planning were obtained from the University of California Santa Cruz (UCSC) database and primers were designed with web-based primer programs Exon Primer and Primer3. Primers were blasted to the genome using UCSC blat.

All exons and flanking introns were analysed by Sanger sequencing from *BCAR3* (Study I), *LRP5* (Studies I-IV), *LRP6* and *PTHLH* (Study III), and *COL1A1* and *COL1A2* (Study IV). DNA was amplified by polymerase chain reaction (PCR) using either AmpliTaq Gold or Phusion High Fidelity DNA polymerase and

purified according to the manufacturer's recommendations before BigDye-labelling and sequencing.

Sequence analysis (Studies I-IV)

Sequence analysis was performed with the Sequencher program. Identified sequence variants were compared with public genomic databases: University of California Santa Cruz (UCSC), National Center for Biotechnology Information (NCBI), 1000 genomes and National Heart, Lung and Blood Institute (NHLBI*). In silico* predictions regarding changes in protein function from sequence variants were performed with web-based software PolyPhen2 and SIFT, and predictions on changes in RNA splicing were performed with NNSPLICE0.9 and NetGene2.0. Control samples were screened by Sanger sequencing, except for the Turkish control samples in Study II, in which the investigated nucleotide change was excluded by digestion of PCR fragments by restriction enzyme *HphI* and gel electrophoresis on a 1.2% agarose gel with ethidium bromide staining.

Exon trapping (Study II)

Appropriate tissue samples for RNA extraction were unavailable for two patients in Study II. The consequences of their splice site mutations were therefore analysed using the Exon Trapping System (Buckler et al., 1991). PCR amplified fragments of patient DNA were cloned into the PCR 2.1-TOPO Vector, subcloned into the exon trapping vector, pSPL3, and transfected by lipofection into COS-7 cells. After 24 hours of incubation, total RNA was isolated, cDNA was synthesized, and PCR amplified. The RT-PCR products were visualized on 1.2% agarose gels by ethidium bromide staining.

Microsatellite studies (Study I)

A genome-wide microsatellite screening was performed at the Institute for Molecular Medicine Finland (FIMM), Helsinki, Finland, with a panel of 384 polymorphic fluorescent markers evenly spread over the 22 autosomes and the X-chromosome according to standard methods. Twenty-four DNA samples from the investigated pedigree were included in the screening (19 clinically assessed family members and 5 additional parents). Microsatellite analysis was performed with GeneMapper. LOD score analyses were performed under the assumption of autosomal dominant inheritance with the presumed disease-gene frequency of 0.01, a penetrance of 90% and a phenocopy rate of 2.4%. Two-point linkage LOD scores were calculated with ANALYZE. For LOD scores detected under homogeneity, the pointwise thresholds set according to Lander and Kruglyak (1995) are P p .000049 (equivalent to a LOD score of 3.3) for significant linkage and P p .0017 (LOD score 1.9) for suggestive linkage. Non-parametric multipoint linkage p-scores were analyzed with Simwalk2 v.2.96 under recessive, dominant, and additive inheritance models with the Kong and

Cox significance testing procedure, where a p-value below 0.001 is considered as significant evidence and a p-score below 0.01 as suggestive evidence of linkage.

Mistyping and haplotype analyses were performed with Simwalk 2. Haplotypes were visualized with the web-based program Haplopainter. A fine-mapping of the linkage areas was executed with 45 additional markers spaced 1 cM chosen according to position and heterogeneity.

Genome-wide SNP microarray (Study I)

The work was performed at FIMM. DNA from all family members was screened with an Illumina 610K-quad microarray chip according to the manufacturer's protocol. Genotype calling and CNV analysis were performed with Illumina software BeadStudio.

Target enrichment and deep sequencing (Study I)

The work was performed at FIMM. Target enrichment and deep sequencing was performed on DNA from three affected family members. The biotinylated RNA oligomer ("bait") library was planned with the net-based program eArray based on genomic data from the UCSC database. It was designed to, within the linkage areas, cover all exons with 40 bases of flanking introns and all CpG islands, which are possible promoter areas containing multiple methylation sites. All baits were 120 bases long and manufactured for paired-end sequencing. The baits were planned for 3 x tiling, which means that three separate baits covered each base in each interval. Bait sequences were blasted to the genome and after removal of promiscuous probes (approx. 5%), which could attach to more than one location in the genome, 15279 baits remained.

The DNA samples were fragmented with ultrasonication (Covaris S220), tagged, and amplified according to the Sure Select instruction manual version 1-2. DNA fragments were thereafter hybridized to the bait library, i.e. the selected genomic regions were captured by the biotinylated baits and separated with magnetic beads. The captured DNA fragments were thereafter amplified by PCR. Fragment size and quantity were ensured by measurements with a microfluidics-based platform (Bioanalyzer) between every step of the protocol.

Next-generation sequencing was performed in a single run with a Solexa sequencer as paired-end reading with a read length of 2x56 bases. The sequence alignment was executed with the Burrows-Wheeler alignment tool (Li *et al*., 2009) and the variant calling with SAMtool's pileup (Li *et al*., 2009). The reference assembly was the human genome version GRCh37/hg19 (http://genome.ucsc.edu). Sequence variants common to all three subjects were compared to known variants in UCSC, NCBI, 1000 genomes and NHLBI databases. We fil-

tered for variants in coding, splice or regulatory regions, which occurred in less than 15% of the general population, as more common genomic loci with an impact on bone health would presumably have been identified in recent large-scale GWASs. The impact of a variant on protein splicing was assessed *in silico* with programs defined in the "Sequence analysis" chapter. The exonic non-synonymous variant deemed to be deleterious to protein function by prediction analysis was investigated by PCR and Sanger sequencing from all family members and from healthy Finnish controls.

Functional studies (Study II)

Functional studies were performed with transient transfection of plasmid constructs into HEK293T-cells. The utilized constructs were: a wild type construct (LRP5-WT_{9L}), a mutant construct containing the investigated mutation (LRP5-p.E528_V529ins21), and a mutant construct (LRP5-Mut_{3L}) containing a deletion of six out of nine consecutive leucine residues (c.43_60del) causing a signalling defect known to hinder normal secretion of the protein. Corresponding constructs were also used for the secretion assay, which were truncated at exon 20 thereby lacking the transmembrane and cytosolic domains (LRPN-WT_{9L}, LRP5N-p.E528_V529ins21 and LRP5-Mut_{3L}). All plasmids contained a myc-epitope enabling immunodetection of LRP5 with an anti-myc antibody.

Assessment of canonical Wnt signalling activity was performed with a dual-luciferase reporter assay. A Firefly luciferase reporter gene construct (Topflash TCF reporter plasmid), which contains TCF binding sites as a target for canonical Wnt signalling activation, and Renilla luciferase vectors, for control of transfection efficiency and normalization of luciferase activity, were co-transfected with the wild-type and mutant constructs. In addition, plasmids encoding Norrin, Frizzled-4 (Fzd4), mesoderm development candidate 2 (MesdC2), and receptor-associated protein (RAP) were co-transfected to optimize intracellular signalling and molecular transport conditions as previously described (Hsieh *et al*., 2003; Ai *et al.*, 2005, Chung *et al.*, 2009). The mutant LRP5-Mut_{3L} served as a positive control for impaired Wnt signal transduction (Chung *et al.*, 2009). Co-transfection with a control vector pcDNA3.1-LacZ was performed to assure that equal amounts of DNA were transfected in each experiment. β-actin and calnexin antibodies served as positive controls for proper separation of cytosolic and membrane proteins. β-actin antibodies and Coomassie staining of a 60-kilodalton (kDa) protein in the conditioned medium were used as controls in the secretion assay for comparable loading of cell lysate and medium (Western blotting).

Table VI. Laboratory tests and radiological examinations, which were utilized for assessment of subjects included in the studies. The extent of examinations was decided individually.

LABORATORY TESTS		
Calcium metabolism		**Study**
P-Ca, U-Ca	calcium	I, III, IV
P-Pi, U-Pi	phosphate	I, III, IV
P-Afos	alkaline phosphatase	I, III, IV
S-Afos -Is	alkaline phosphatase isoforms	I, III, IV
S-D-25	vitamin D	I, III, IV
fP-PTH	parathyroid hormone	I, III, IV
vB-Het-ion	acid-base balance	I, III, IV
P-Crea, U-Crea	creatinine	I, III, IV
U-INTP	n-terminal telopeptide of type I collagen	I, III, IV
S-PINP	aminoterminal peptide of type I procollagen	I, IV
S-ICTP	carboxy-terminal peptide of type I collagen	I, IV
Hormones		
S-LH	luteinizing hormone	I, IV
S-FSH	follicle stimulating hormone	I, IV
S-Estdio / S-testoL	estradiol / testosterone	I, IV
S-IGF-1	insulin-like growth factor 1	I, III, IV
S-T4-f	free thyroxin	I, III, IV
S-TSH	thyroid stimulating hormone	I, III, IV
Other metabolic parameters and samples		
B-GHbA1C	glycosylated haemoglobin	I, IV
fP-Chol	cholesterol (includes total cholesterol, LDL, HDL and triglycerides)	I, IV
P-Alat	alanine aminotransferase	I, IV
P-TT	tromboplastin time	I, IV
P-Urate	urate	I, IV
RADIOLOGICAL EXAMINATIONS		
DEXA	dual energy X-ray absorptiometry (lumbar spine, hip, whole body)	I, II, III, IV
Thorax	(posterior-anterior, lateral)	I, II, III, IV
Thoracal spine	(anterior-posterior, lateral)	I, III, IV
Lumbar spine	(anterior-posterior, lateral)	I, III, IV
Pelvis	(anterior-posterior)	I, II, III, IV
Knees	(anterior-posterior)	I, II, III, IV
Long bones	(left hemiskeleton, anterior-posterior)	I, II, III, IV
Hands	(dorsal-palmar, bone age assessment)	I, II, III, IV

Table VII. Reagents, hardware, and software utilized in the genetic analyses.

DNA / RNA PURIFICATION	
Extraction of genomic DNA from peripheral blood	Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN, USA)
RNA extraction from peripheral blood	PaxGene Blood RNA Extraction Kit (PreAnalytix, Hombrechtikon, Switzerland)
RNA extraction from cultured fibroblasts (Study II)	Versagene RNA Cell Kit (Gentra Systems, Minneapolis, MN, USA)
RNA extraction from cultured COS7-cells (Study II)	RNAeasy Mini Kit (Qiagen, Hilden, Germany)
RT-PCR (for cDNA) (Study II)	OneStep RT-PCR Kit (Qiagen, Hilden, Germany)
DNA and RNA concentration and quality assessment	NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA)
AMPLIFICATION AND SEQUENCING	
primer planning	genomic sequences from UCSC database (http://genome.ucsc.edu/)
	Exon Primer (http://ihg2.helmholtz-muenchen.de/)
	Primer3 (http://frodo.wi.mit.edu/)
PCR amplification and fragment purification	AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA)
	+ ExoSAP-IT (USB, Cleveland, OH, USA)
	Phusion High Fidelity DNA polymerase (Finnzymes, Espoo, Finland)
	+ Quickstep2 PCR purification kit (Edge Bio, Gaithersburg, MD, USA)
PCR fragment labelling for sequencing	BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems)
sequencing	ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA)
SEQUENCE ANALYSIS	
sequence analysis	Sequencher 4.7 and 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA)
comparison to known genomic variants	UCSC (http://genome.ucsc.edu/)
	NCBI (http://www.ncbi.nlm.nih.gov/)
	1000 genomes ((http://www.1000genomes.org)
	NHLBI exome variant server (http://evs.gs.washington.edu/EVS/)
in silico splice prediction	NNSPLICE0.9 (http://www.fruitfly.org/)
	NetGene2.0 (http://www.cbs.dtu.dk/services/NetGene2/)
protein function prediction	SIFT (http://sift.jcvi.org)
	PolyPhen2 (http://genetics.bwh.harvard.edu/pph2)
EXON TRAPPING ANALYSIS	
exon trapping analysis	Exon Trapping System (Gibco BRL, Gaithersburg, MD, USA)
vectors	PCR 2.1-TOPO Vector (Invitrogen, Carlsbad, CA, USA)
	pSPL3 (Gibco BRL, Gaithersburg, MD, USA)
MICROSATELLITE ANALYSIS	
DNA sizing and allele calls	GeneMapper 4.0 (Applied Biosystems, Foster City, CA, USA)
two-point LOD score analysis	ANALYZE (Hiekkalinna T, et al. Twin Res Hum Genet 2005;8:16-21)
multipoint LOD score analysis and haplotyping	SimWalk2 (Sobel E, et al. Am J Hum Genet 2002;70:496-508)
haplotype visualization	Haplopainter v.029.5 (http://haplopainter.sourceforge.net)
GENOME-WIDE SNP MICROARRAY AND CNV	
Single nucleotide polymorphism microarray	Illumina Human610K-quad chip (Illumina Inc., San Diego, CA, USA)
variant calling and CNV analysis	BeadStudio (Illumina Inc.)
TARGET ENRICHMENT AND MASSIVE PARALLELL SEQUENCING	
target enrichment bait library planning	eArray (https://earray.chem.agilent.com/earray/)
DNA fragmentation	Covaris S220 (Covaris, Inc., Woburn, MA, USA)
targeted DNA enrichment	Sure Select Target Enrichment System, version 1_2
	(Agilent Technologies Inc., Santa Clara, CA, USA)
fragment quality assessment	Agilent 2100 Bioanalyzer (Agilent Technologies Inc.)
massive parallell sequencing of enriched fragments	Solexa (Illumina, San Diego, CA, USA)
fragment blasting and variant calling	Burrows-Wheeler alignment; SAMtool's pileup (Li *et al*., 2009)

5. RESULTS AND DISCUSSION

Study I
Clinical and genetic findings in a family with primary osteoporosis

The probands were two young brothers (Figure 3, individuals 14 and 15), who presented with recurrent arm fractures and multiple vertebral compression fractures (VCFs) in the thoracic spine. They had osteoporotic lumbar spine (LS) BMD Z-scores (-3.1 and -2.7) (Table VIII). There was a high prevalence of osteoporosis in maternal relatives and no history of osteoporosis or proneness to fractures in the paternal family members. The maternal grandfather and two of his siblings suffered from VCFs and severe kyphosis. Of the 19 family members assessed, eleven were regarded as affected with osteoporosis. The phenotype status was set as "unknown" for family members not fulfilling the criteria set for affected individuals, as most subjects were quite young and may develop a disease phenotype with time. The father of the probands, who had never sustained a fracture or suffered from back problems, was found to have a low LS BMD Z-score (-2.5). As the parental origin of the probands' phenotype was uncertain, they were also classified as "unknown". The clinical and radiologic features of the family members are summarized in Table VIII.

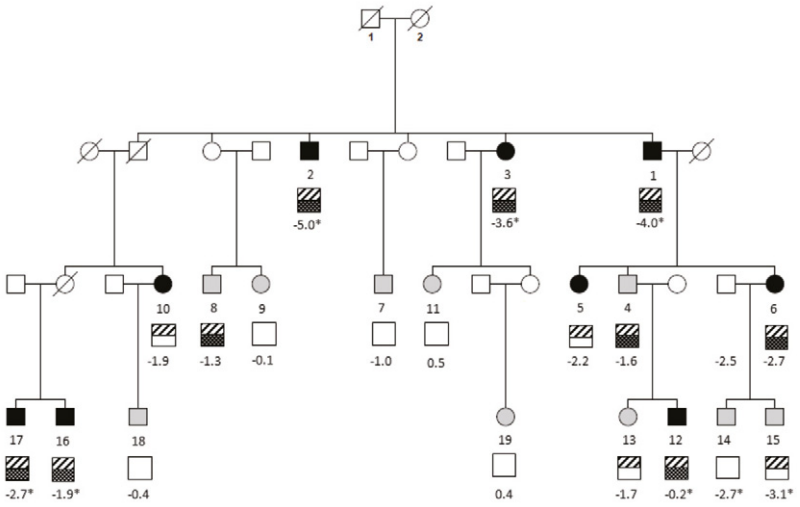


Figure 3. Family tree of the family with a high prevalence of early-onset osteoporosis. Affected individuals are marked in black, unaffected/uncertain in grey and unassessed in white. The disease-associated haplotypes of the two identified genomic loci with positive linkage evidence are shown in boxes under each genotyped individual (striped box: 1p21.3-p22.2; checkered box: 11q22.1-q22.3; empty box: non-carrier). Lumbar spine BMD T-/Z-scores according to Table VIII are shown below the boxes. Individuals with vertebral compression fractures are marked with an asterisk.

Table VIII. Clinical data for the family with a high prevalence of early-onset osteoporosis. Subjects are numbered according to that of Figure 3. BMD values are presented in T-scores for subjects over the age of 50 years and in postmenopausal women (values bolded) and in Z-scores for the others. The most severe vertebral changes found in spinal X-rays are presented according to Genant grading in adults (capitals) and according to Mäkitie in children. Probands are marked with italic font.

Subject	Sex	Age (y)	Peripheral fractures (n)	Age at first fracture (y)	BMD T- (bolded) or Z-score			vertebral changes	Loss of adult height (cm)
					lumbar	hip	whole body		
Affected									
1	M	65	4	33	**-4.0**	**-1.4**	**-2.3**	3C	11
2	M	62	3	7	**-5.0**	**-3.1**	**-3.8**	3C	12
3	F	59	8	5	**-3.6**	**-1.5**	**-3.0**	3C	4
5	F	33	0		-2.2	-1.5	-2.0	0	0
6	F	40	0		-2.7	-1.7	n/a	0	0
10	F	53	1	38	**-1.9†**	**-2.1**	**-2.1**	0	0
12	M	11	0		0.2	-0.2	0.4	2a/3a	0
16	M	35	1	21	-1.9	-1.2	-2.5	3C	2.5
17	M	33	10	7	-2.7	-0.7	-2.2	3C	1.5
Status set as unknown in genetic analysis									
4	M	41	0		-1.6	-0.3	-0.4	0	0
8	M	31	2	18	-1.3	-1.1	-1.7	0	0
13	F	13	0		-1.7	0.4	-0.5	0	0
14	*M*	*12*	*4*	*8*	*-2.7*	*-2.0*	*-2.3*	*2a*	*0*
15	*M*	*9*	*2*	*8*	*-3.1*	*-1.8*	*-2.2*	*3b*	*0*
7	M	53	0		**-1.0**	**-0.8**	**-1.6**	0	0
9	F	41	1	12	-0.1	-0.3	0.7	0	0
11	F	34	0		0.5	0.4	0.5	0	0
18	M	30	2	16	-0.4	-0.3	-0.1	0	0
19	F	19	0		0.4	1.2	1.0	0	0

†Subject 10 had osteoporotic BMD values prior to our study and had received bisphosphonate treatment for several years.

Clinical and radiographic findings

The affected subjects had no features of OI nor any distinct facial or body features. They presented normal joint laxity, their hearing and teeth were normal, and they had white sclerae. In juvenile subjects, bone age was appropriate for chronological age confirming normal skeletal maturation. All individuals were of normal height. There was a high frequency of peripheral fractures from low-impact trauma among affected individuals. The radiographic features included poor mineralization of the long bone metaphyses, but normal cortical width and shape in the diaphyses. The first and most severely affected skeletal region was the thoracic spine. We found anterior vertebral wedging in a young subject. The wedging had progressed into complete VCFs in older individuals, and patients in their 60s had severe VCFs in virtually all vertebrae, which caused significant kyphosis and an adult height loss of up to 12 cm. The severity of symptoms and findings varied between the affected subjects; the findings tended to be more severe in males. The VCFs seemed to develop gradually with no pain or other symptoms, apart from subject 3 who had experienced several painful VCFs. Spinal X-rays of the affected subjects at different ages are presented in Figure 4.

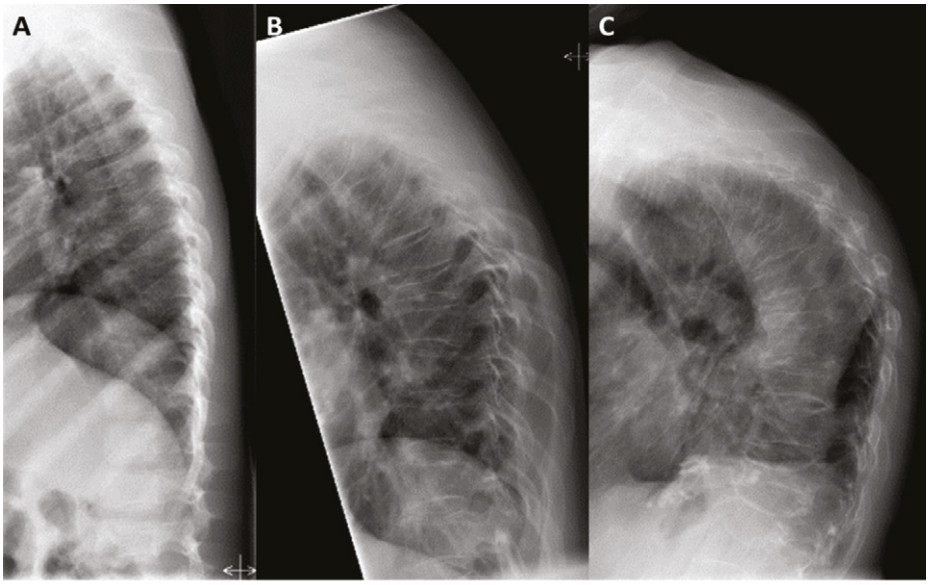


Figure 4. A. A 12-yearold male (12) had wedging of thoracic vertebrae grade 2b to 3b. B. A 33-yearold male (17) exhibited multiple VCFs grade 3C. C. This 62-yearold male (2) had severe VCFs (grade 3C) in virtually all visible vertebrae.

Biochemical findings

All markers of calcium and phosphate homeostasis were normal, however one affected subject demonstrated mild hypovitaminosis D. No patients had hypercalciuria or hyperparathyroidism. All measured markers of bone formation and degradation were within normal age-specific ranges. Exclusion of secondary causes of osteoporosis was performed by clinical examination and appropriate laboratory tests.

Histomorphometric findings

In the probands (14, 15), the histology and histomorphometry based on transiliac bone biopsies showed low bone volume, thin bone trabecuale and a normal or predominantly resorptive bone turnover (Figure 5). The lamellar structure of the bone was normal based on polarizing microscopy, but mineralization rates were decreased. One young male (12) had normal bone volume, but clearly decreased trabecular thickness (-3 SD) compensated for by an increased number of trabeculae. His bone turnover and mineralization rate were normal. One adult male (17) had severe osteoporosis. His bone trabeculae were thin and scarce, and bone turnover was dominated by resorption. The mineralization rate was normal. (Table IX)

Table IX. Bone histomorphometry findings in four patients with hereditary osteoporosis. The numbers (#) in parentheses correspond to the numbering in Figure 3 and Table VIII. Values low for sex and age are noted in bold, and high values are underlined.

Parameter	Male, 11 years (#12)	Male, 33 years (#17)	Male, 9 years (#15)	Male, 12 years (#14)
BV/TV (%)	23.6 (24.4 ± 4.3)	**11.1 (22.0 ± 3.9)**	**13.8 (24.4 ± 4.2)**	**9.2 (24.4 ± 4.3)**
Tb.N (/mm)	3.1 (1.66 ± 0.22)	1.3 (1.7 ± 0.4)	1.8 (1.78 ± 0.17)	**1.3 (1.66 ± 0.22)**
Tb.Th (μm)	**77.1 (148 ± 23)**	**88.4 (138 ± 24)**	**78.0 (129 ± 17)**	**69.0 (148 ± 23)**
O.Th (μm)	6.4 (6.7 ± 1.7)	6.4 (9.7 ± 4.6)	6.3 (5.9 ± 1.1)	9.1 (6.7 ± 1.7)
OS/BS (%)	**13.5 (22.1 ± 7.8)**	**5.6 (14.0 ± 4.6)**	**13.8 (29.1 ± 12.9)**	**13.7 (22.1 ± 7.8)**
OV/BV (%)	1.7 (2.1 ± 1.0)	**0.8 (3.5 ± 1.9)**	2.2 (2.6 ± 1.0)	1.8 (2.1 ± 1.0)
Ob.S/BS (%)	3.5 (6.7 ± 4.5)	**1.9 (6.0 ± 1.1)**	4.0 (8.2 ± 4.4)	6.5 (6.7 ± 4.5)
ES/BS (%)	**4.6 (14.9 ± 5.6)**	5.5 (4.5 ± 1.9)	12.8 (17.0 ± 6.0)	**3.9 (14.9 ± 5.6)**
Oc.S/BS (%)	0.8 (0.9 ± 0.4)	3.4 (0.6 ± 0.4)	4.2 (1.3 ± 0.6)	0.5 (0.9 ± 0.4)
MAR (μm/day)	N/A	0.5 (0.6 ± 0.3)	**0.5 (0.9 ± 0.1)**	**0.6 (0.9 ± 0.1)**

BV/TV, bone volume/tissue volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; O.Th, osteoid thickness; W.Th, wall thickness; OS/BS osteoid surface/bone surface ; OV/BV, osteoid volume/bone volume; Ob.S/BS, osteoblast surface/bone surface; ES/BS, eroded surface/bone surface; Oc.S/BS, osteoclast surface/bone surface; MAR, mineral apposition rate. N/A: not available. Age- and sex-specific reference values are given for each parameter in parentheses (mean ± SD). (for children: Glorieux *et al.*, 2000; for the adult: Rehman *et al.*1994)

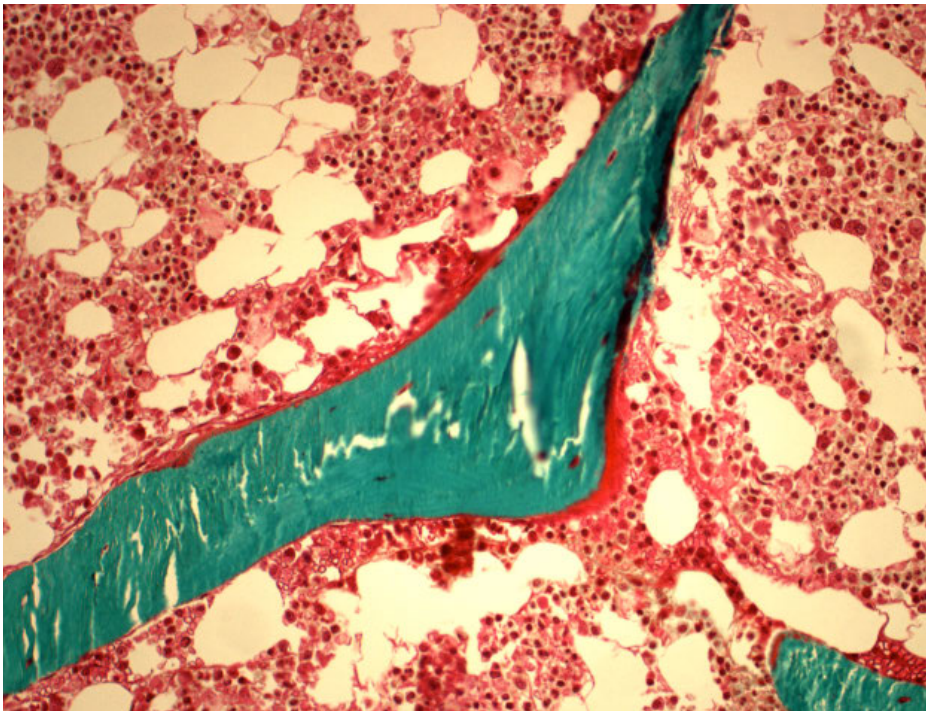


Figure 5. Histomorphometry of a bone sample from a 12-yearold boy (14). A modified Masson-Goldner trichrome stain was used (bone, green; osteoid, red). Bone trabeculae are thin and scarce, but the amount of osteoid is normal.

Genetic results

Multipoint non-parametric linkage (NPL) analysis using an additive model revealed p-values of suggestive linkage in 1p22.1 at adjacent markers D1S2868 (p=0.0040) and D1S206 (p=0.0054). (Kong and Cox, 1997) After fine mapping, recombinations at D1S435 and D1S2819 in individuals 10 and 12 restricted the area to a 4.9 Mb region at 1p21.3-p22.2. In chromosome 11, marker D11S4175 had a NPL p-value of 0.0328. Based on haplotype analysis, this area was included in the fine mapping. Thereafter the lowest NPL p-value was 0.007 at D11S898 in 11q22.1, and the shared region stretched from marker D11S4210 to D11S2000 (11q22.1-q22.3). Two-point LOD-scores were assessed and haplotype analysis of all chromosomes was performed, but since no family members were classified as healthy in the analysis, the statistical strength of the two-point LOD-score analysis suffered and the best correlation between phenotypes and haplotypes was found with the multipoint NPL analysis. No shared haplotype was identified in the X-chromosome (data not shown). Linkage analysis results are visualized in Figure 6. Neither of the presented regions of interest could alone explain the phenotype of the family. Genomic deletions segregating according to the haplotype analysis, or shared by the probands and their father, were excluded by a microarray CNV analysis. As the inheritance based on the

linkage analysis was suggestive of multigenic origin, all exons in the identified regions were sequenced in order to look for putative disease-causing mutations.

Target enrichment and next-generation sequencing analysis was performed on three affected individuals (1, 2 and 17); they shared 66 heterozygous sequence variants within the targeted regions. All variants could be found in public databases. Since the disease-causing changes in this pedigree are presumably clinically significant, SNPs which occur in less than 15% of the general population according to data from the 1000 genomes database were filtered. It was postulated that recent large-scale GWASs would have identified more common loci with effect on variations in BMD and proneness to fracture (Rivadeneira *et al*., 2009; Estrada *et al*., 2012). Out of the remaining 18 variants, only five resided in coding regions, near splice sites, or in regulatory regions. One was a synonymous SNP and one an intronic variation residing seven bases downstream of a splice site. Neither variation was predicted *in silico* to alter protein splicing. The three remaining SNPs were missense variants, which resided in *breast cancer anti-estrogen resistance 3* (*BCAR3*) in chromosome 1, and in *matrix metalloproteinase 10* (*MMP10*) and *dynein, cytoplasmic 2, heavy 1* (*DYNC2H1*) in chromosome 11. Only the change in *BCAR3* was predicted to be deleterious to protein function. Next generation sequencing artifacts and correct allele distribution according to haplotype analysis were confirmed by Sanger sequencing of this region in *BCAR3* from DNA samples of all family members and 60 controls. This variant could be found at a low frequency (3.1%; 5/160 alleles) in healthy Finnish controls. (Table X)

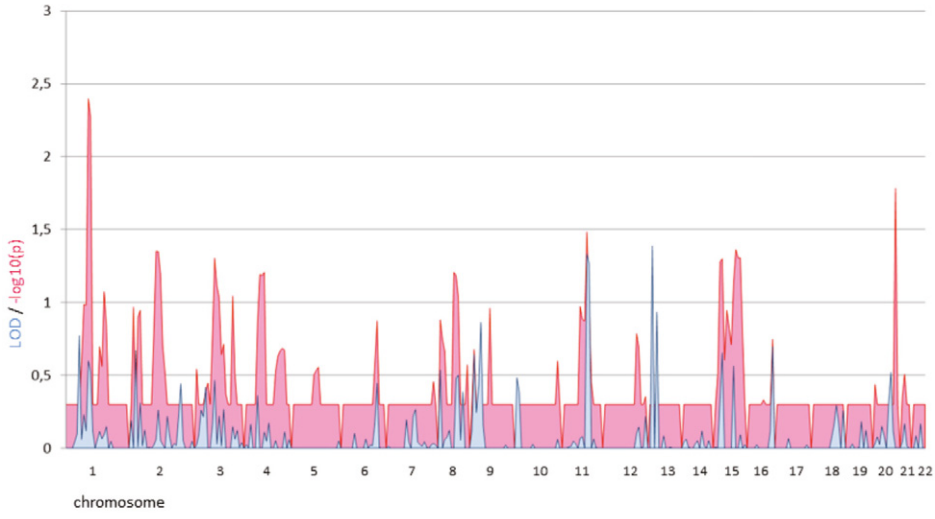


Figure 6. Plot of two-point LOD-scores (blue) and multipoint non-parametric linkage p-scores presented as –log10(p) values (pink; Kong&Cox statistics, additive model) for chromosomes 1-22.

Table X. The results from the genetic analysis performed on a pedigree with a high prevalence of osteoporosis. The affected family members largely shared two genomic regions based on microsatellite linkage and haplotype analysis. The targeted next-generation sequencing of these linkage areas revealed five shared heterozygous variations, three of which were non-synonymous, after filtering of results. LOD: maximum two-point LOD-score; NPL: lowest multipoint non-parametric linkage p-value; MAF: minor allele frequency in the general population based on the 1000 genomes database.

Region	LOD	NPL	SNP n:o	Gene	Variant consequence	MAF
1p21.3-p22.2	0.8	0.004	rs151322895	*BCAR3*	F6Y	0.3%
				Breast cancer anti-estrogen resistance 3	probably damaging	
11q22.1-q22.3	1.33	0.007	rs3809018	*MMP27*	V178V	12.3%
				Matrix metalloproteinase 27	splicing unaltered	
			rs486055	*MMP10*	R53K	6.2%
				Matrix metalloproteinase 10	benign	
			rs12146610	*DYNC2H1*	Q304L	4.2%
				Dynein, cytoplasmic 2, heavy chain 1	benign	
			rs34491690	*CARD17*	Intronic, near splice site	8.0%
				Caspase recruitment domain family, member 17	splicing unaltered	

To conclude: The investigated family was identified based on two severely affected brothers, who had sustained multiple peripheral and vertebral fractures. Upon assessment of 19 family members the inheritance seemed autosomal dominant. Affected family members suffered from mild VCFs in childhood and low BMD in adulthood. With increasing age, the affected individuals sustained severe VCFs of thoracic, and later lumbar, vertebrae. No other phenotypic features could be identified and bone turnover markers were normal. OI was excluded clinically and based on linkage analysis, and no mutations could be found in *LRP5.* Two regions with NPL p-score indicative of suggestive linkage were identified in 1p21.3-p22.2 and 11q22.1-22.3, none of which alone could explain the clinical phenotype. One of the probands did not carry either of the regions shared by affected maternal relatives. His father also had low BMD-values, but the phenotype of the boy resembled more that of maternal family members. Next-generation sequencing of all exons and flanking introns in the identified regions revealed three non-synonymous variations, one of which was predicted to damage protein function. This variation occurs at a low frequency in the general population.

In the most significant linkage area, in **chromosome 1**, the only missense variation common to most affected patients resided in *BCAR3. BCAR3* is a gene involved in anti-estrogen resistance in breast cancer treatments (van Agthoven *et al*., 1998). The significance of this gene with regard to bone health is unclear. In **chromosome 11**, both discovered variants were predicted to be benign. The variant in *MMP10* has, however, been linked to the risk for ligament rupture in the knee, and the gene has been implicated in osteoarthrosis studies, indicating a role in cartilage and ligament maintenance (Kevorkian *et al*., 2004; Posthumus *et al*., 2011). *DYNC2H1* is involved in protein transportation in the primary cilium, which is also the mechanosensory organ of the osteocyte. Biallelic mutations in *DYNC2H1* cause polydactyly-short-rib syndrome, but the variant identified here has not been linked to this disease; the phenotype in heterozygotes is not known (Hoey *et al*., 2011; Merrill *et al*, 2009; Dagoneau *et al*., 2009). Future functional studies are needed to clarify the significance of the discovered variations with regards to bone homeostasis.

Study II
LRP5 mutations affecting splicing

OPPG is a rare autosomal recessive syndrome caused by loss-of-function mutations in *LRP5*.

The phenotype of our patients is typical for OPPG: loss of vision in infancy and severe symptomatic osteoporosis presenting in childhood as VCFs, long bone low-impact fractures, and bowing. About 25% of reported cases show mental retardation (Ai *et al.*, 2005).

All of our patients are congenitally blind with diagnoses of exudative retinopathy (Patient 1), retrolental fibroplasia (Patients 2, 3A, and 3B) and bilateral cataract, right microphthalmia, left retinal calcifications, and absent visual evoked potentials (Patient 4). Patient 2 has severe mental retardation and epilepsy; the other patients have normal psychomotor development. Patient 1 has unrelated parents of Eastern European origin and Patients 2-4 consanguineous parents from the Middle East. Their bone phenotypes are described separately below.

Clinical findings

Patient 1 is a 13 year-old girl, who sustained a lower leg fracture at 2 and a forearm fracture at 5 years of age. The clinical diagnosis of OPPG was set at age 5 when she was diagnosed with multiple VCFs and severe osteoporosis (LS BMD Z-score -3.8). From 6 to 10 years of age she received bisphosphonate treatment with dinatriumpamidronate 1.0 mg/kg/day for three consecutive days every four months. Her LS BMD Z-score increased to -1.6 and spinal compression fractures improved with treatment. According to her parents, the medication also improved her strength and physical well-being. Her BMD and clinical status have remained stable after discontinuation of bisphosphonate treatment. Both parents are osteopenic (LS BMD Z-score -2.1) and their vision and eye examinations are normal. The mother has sustained one forearm fracture.

Patient 2 is a 13-year-old mentally retarded boy with complex partial seizures well-controlled with anticonvulsant medication. His brain MRI is normal. After sustaining four femoral fractures between 3 and 7 years of age he has not been able to walk independently. He receives bisphosphonate therapy (alendronate 70 mg orally once a week) since three years. His LS BMD Z-score has improved from -2.0 to -1.4 and the total body BMD Z-score from -3.3 to -2.4. He has not sustained fractures while on medication and is now able to walk with support. Both parents have osteopenia, use eyeglasses for reading but report no other visual problems. The father has adult-onset diabetes treated with insulin.

Patients 3A and 3B are young adult brothers of normal intelligence. They have been wheelchair-dependent since adolescence due to skeletal deformities from multiple low-impact fractures (radius, humerus, and/or the hip, from age 11 and 7 years, respectively). They have severe osteoporosis; the current LS BMD Z-score of patient 3A is -5.3. The father (age 55) has osteoporosis (LS BMD Z-score -3.1), and the mother (age 55) is osteopenic.

Patient 4 is a 17-year-old male who suffers from bilateral tibial bowing and VCFs since the age of 6. He has no history of peripheral fractures. He can stand unsupported but needs support when walking, mainly due to visual problems. A recent radiographic skeletal survey revealed generalized osteoporosis, scoliosis, dorsal kyphosis, severe flattening and biconcave appearance of the vertebrae, and bowing and deformation of the long bones.

Genetic findings and functional studies

Sanger sequencing of *LRP5* revealed that **Patient 1** is a compound heterozygote for a missense mutation c.1067C>T (S356L) in exon 6 and a splice site mutation c.4112-2A>G (intron 19). The patient's mother is heterozygous for the c.1067C>T, and the father for the c.4112-2A>G mutation. The mutant protein encoded by the allele harboring the S356L mutation is known to traffic normally but is unable to transduce Wnt1 and Wnt10b signals (Ai *et al.*, 2005). Since no tissue samples or RNA from this patient were available to confirm that the c.4112-2A>G splice site mutation impaired splicing, a heterologous splicing assay was undertaken (Figure 7). Patient DNA fragments were inserted into the cloning site of a pSPL3 plasmid. This plasmid contains a multiple cloning site within an intron, which is flanked by splice donor and acceptor sites of two open reading frames (Buckler *et al.*, 1991). In the absence of an inserted fragment, transfected cells remove the endogenous intron to generate a vector/vector product of 263 base pairs (bp) (Figure 7A, lane 6; Figure 7B, α splicing product). In the presence of an inserted wild-type fragment a spliced vector/genomic product containing the inserted fragment is produced in addition to the vector/vector product of 263 bp (Figure 7A, lane 2; Figure 7B, β splicing product). Mutant constructs did not produce any detectable vector/genomic product from the mutant insert obtained from Patient 1 (Figure 7A, lane 3), which indicates that the substitution of the splice acceptor site (AG to GG) of intron 19 promotes splicing out of exon 20 and adjoining introns without activation of cryptic splice sites. The in-frame splicing out of exon 20 in Patient 1 does not cause a stop codon, but completely removes the transmembrane domain of the protein.

Patient 2 carries a homozygous splice site mutation c.1015+1G>T (intron 5), and both parents are confirmed to be heterozygous for the mutation. The heterologous splicing assay of this mutant construct, containing a substitution in the donor splice site from GT to TT, showed that exon 5 with its adjoining introns is consistently spliced out. This is illustrated by the 263 bp vector-vector product in

Figure 7A (lane 5), as opposed to the presence of a 395 bp vector/genomic product produced by the vectors containing the wild-type exon 5 fragment (Figure 7A, lane 4). The in-frame splicing out of exon 5 in Patient 2 generates a premature stop codon at amino acid position 295, created by the junction of exons 4 and 6. The patient's mental retardation and severe phenotype could be thought to derive from the truncation of both alleles at the start of exon 5. The majority of mutations in *LRP5* which have been linked to mental retardation induce truncation of the protein (Ai *et al.*, 2005). On the other hand, normal cognitive development has been reported in other homozygous OPPG patients with severe protein truncations, and mental retardation has been reported in both homozygous and compound heterozygous OPPG patients (Ai *et al.*, 2005). This indicates that additional factors influence mental development and no specific domain with regards to genotype-phenotype correlation and mental retardation can be found (Ai *et al.*, 2005).

Patients 3A and 3B are homozygous for a splice site mutation c.1584+4A>T downstream of exon 7 in *LRP5*. The mutation was present in the heterozygous state in the patients' parents and was absent in 50 control samples. Splice site mutations at position +4 are rare, accounting for less than 2% of all donor-site mutations (Krawczak *et al.*, 2007). According to *in silico* splice site analyses the wild-type splice donor site has a splice prediction score of 0.99 (the maximum value being 1.00), whereas the splice score of the mutated sequence c.1584+4A>T is only 0.44. RT-PCR revealed that the mutation inactivates the original splice site. This induces the activation of a cryptic splice site 63 basepairs downstream of the original splice site (Figure 8A) and an in-frame insertion of 21 amino acids (p.E528_V529ins21) from intron 7 after the amino acids encoded by exon 7. The wild-type sequence is correctly spliced in a control sample (Figure 8B).

To assess the activity of the canonical Wnt-pathway with regards to the splice site mutation, a dual-luciferase reporter assay (Topflash) was undertaken. The Firefly luciferase reportergen construct, which reflects activation of canonical Wnt signalling, was co-transfected with the wild-type (LRP5-WT_{9L}) and mutant (LRP5-p.E528_V529ins21) constructs, and with plasmids which optimize signalling and molecular transport conditions. Renilla luciferase vectors were transfected for control of transfection efficiency and normalization of luciferase activity. The mutant construct LRP5-Mut_{3L} served as a positive control for impaired Wnt signal transduction. Activities of the mutant LRP5-p.E528_V529ins21 were significantly lower than those of LRP5-WT_{9L} (p values <0.001 in a two-sided Student's t-test calculation) indicating a disturbance in the activation of the Wnt signaling cascade. The c.1584+4A>T splice site mutation of the LRP5 receptor severely impaired signal transduction (Figure 9A).

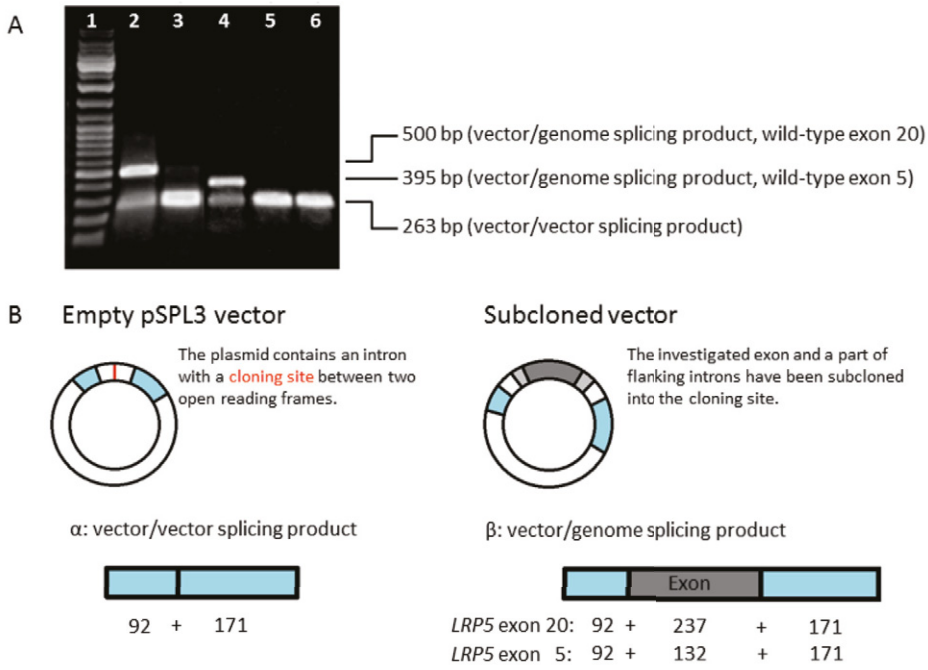
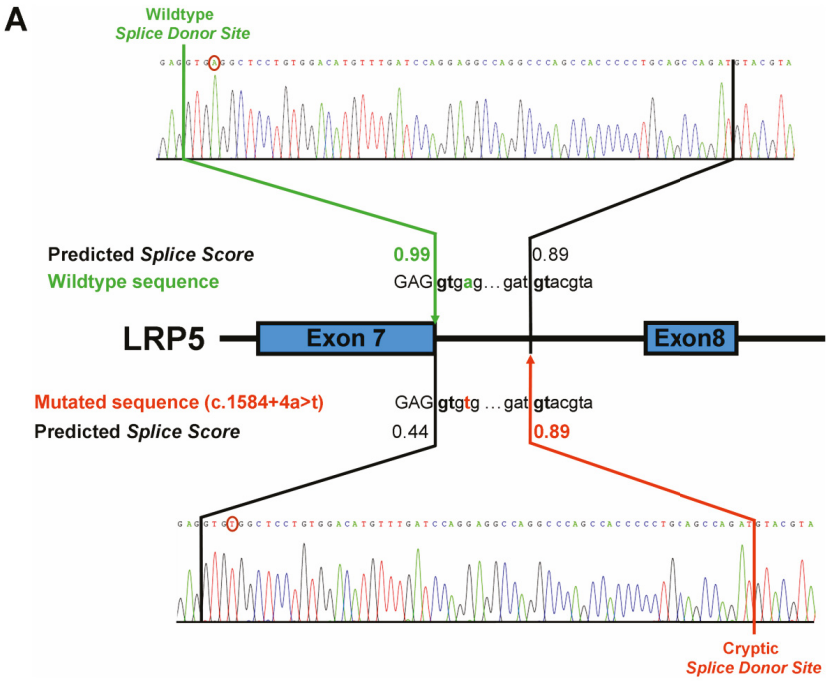


Figure 7. Splicing assay with the Exon Trapping system.

(A) cDNA from COS-7 cells containing wild-type and mutant constructs (exon 20 from Patient 1 and exon 5 from Patient 2) was PCR amplified and visualized on an ethidium bromide gel. Lane 1 contains a DNA sizing marker. Splicing products from constructs are visualized on lanes 2 to 6: Lane 2: wild-type exon 20; Lane 3: mutant exon 20; Lane 4: wild-type exon 5; Lane 5: mutant exon 5; Lane 6: empty pSPL3-plasmid. A vector/vector splice product of 263 bp is produced in the absence of an inserted fragment (A: Lane 6, B: α splice product). Splicing of the vector gives rise to two products in the presence of an inserted wild-type fragment: an abundance of correctly spliced vector/genomic product containing the inserted exon (237 or 132 bp; A: Lanes 2 and 4, B: β) and a smaller amount of the "empty" vector/vector product of 263 bp. No detectable vector/genomic product could be retrieved from mutant constructs containing the inserts obtained from Patients 1 and 2 (Lanes 3 and 5) indicating complete splicing out of exons 20 or 5.

(B) A schematic description of the exon trapping vector, pSPL3, and genomic DNA constructs: intronic vector DNA (white), exonic vector DNA (blue), intronic genomic DNA (light grey) and exonic genomic DNA (dark grey) are indicated. The two possible splicing pathways are shown with their respective spliced products: α (vector/vector) and β (vector/genome).



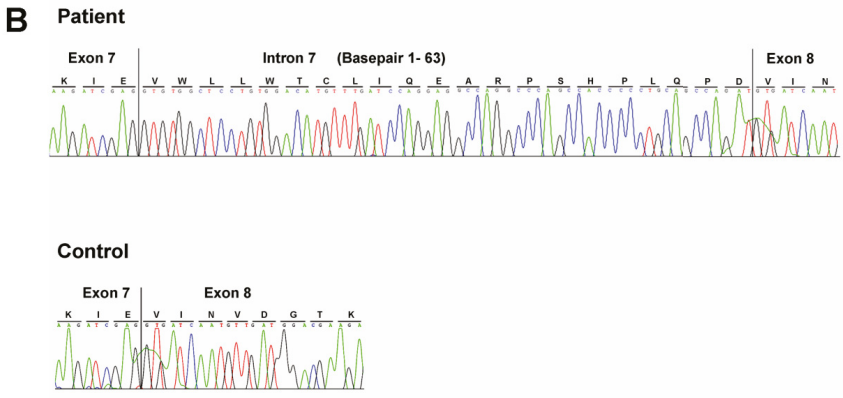


Figure 8. Identification of the c.1584+4A>T disease-causing splice site mutation in *LRP5*.

(A) Genomic DNA amplicons of the *LRP5* exon 7 splice donor site assessed by Sanger sequencing in a control individual (wild-type sequence) and Patient 3A (mutated sequence). Based on *in silico* analyses of the sequence, the predicted splice score decreases from 0.99 in the wild-type splice donor site to 0.44 in the mutated sequence inducing the activation of a cryptic splice donor site downstream.

(B) Electropherograms of *LRP5* cDNA amplicons from Patient 3A (upper panel) and a control individual (lower panel) revealing a mutated splice donor site after exon 7. The patient cDNA sample contains 63 additional nucleotides of intron 7 inserted into the mRNA molecule of exon 7 in the patient sample, whereas the control sample is correctly spliced.

To ensure the validity of the transfections and protein stability, a Western blot of cell lysates from transiently transfected HEK293T cells used in the dual-luciferase reporter assay was undertaken. Immunodetection of the plasmids with an anti-myc antibody confirmed stable expression of both wild-type and mutated LRP5 receptors (Figure 9B). β-actin antibodies served as loading controls for equal amounts of cell lysates.

To determine whether the mutated LRP5 receptor was correctly inserted into the membrane of the endoplasmatic reticulum (ER), the membrane fraction from the cytosolic fraction of transiently transfected cells was separated. Both wild-type and mutated LRP5 were almost exclusively detectable in the membrane fraction (Figure 9C), which indicated correct insertion of the mutated protein into the ER membrane. To further track the molecular basis of the LRP5 loss-of-function, a secretion assay was performed with truncated constructs, which lack the transmembrane and cytosolic domains. The wild-type protein construct was secreted into the medium when normally trafficked within the cell, as confirmed by Western blotting, but the mutant was almost completely absent from the medium (Figure 9D) as was the LRP5N-Mut_{3L}-construct serving as negative control. Our experimental data thereby indicates that the 21 amino acid insertion causes a disruption of the intracellular protein trafficking of the receptor. This may be the consequence of a conformational change in the receptor interfering with molecular interactions required for proper transport to the cytoplasmic membrane and would explain the severely reduced signal transduction observed for the mutant LRP5 receptor in the luciferase reporter assay.

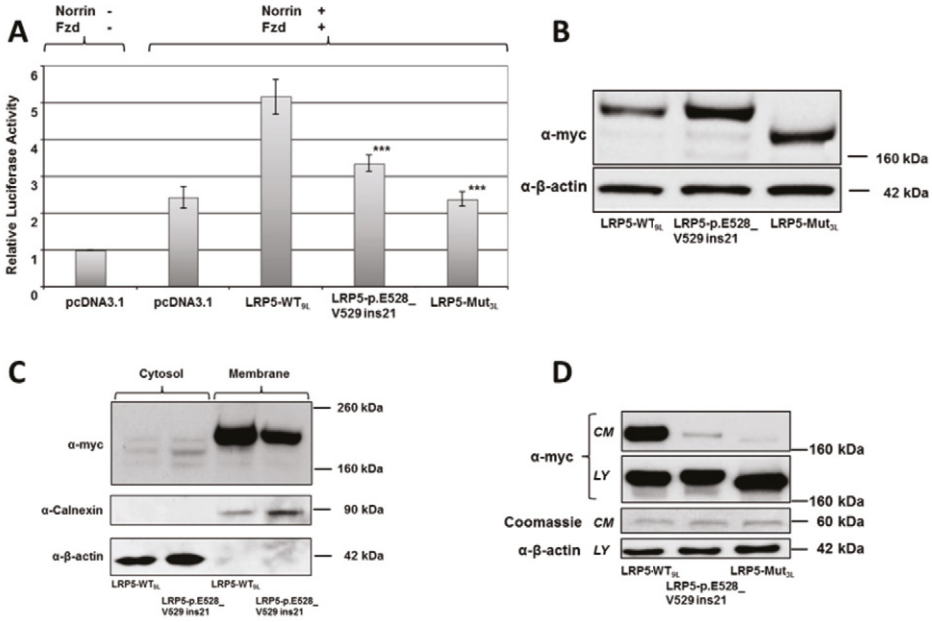


Figure 9. Functional studies with mutant LRP5 protein.

(A) Assessment of the canonical Wnt signalling activity with a dual-luciferase reporter assay. Relative luciferase activities (average and standard deviation) of empty vector (pcDNA3.1), wild-type LRP5 (LRP5-WT_{9L}), and the mutant LRP5-p.E528_V529ins21 are depicted in the graph. The mutant construct LRP5-Mut_{3L} served as a positive control for impaired Wnt signal transduction.

(B) Normal expression of LRP5-WT_{9L} and of the two mutated LRP5 receptors were confirmed by Western blot of cell lysates from transiently transfected HEK293T cells. All plasmids contain a myc-epitope enabling immunodetection of LRP5 with an anti-myc antibody.

(C) Cell fractioning assay of HEK293T cells transiently transfected with LRP5-WT_{9L} or LRP5-p.E528_V529ins21. Calnexin and β-actin antibodies served as positive controls for proper separation of cytosolic and membrane proteins. Both LRP5-WT_{9L} and p.E528_V529ins21 are almost exclusively detectable in the membrane fraction.

(D) Secretion assay by Western blotting of conditioned medium (CM) and cell lysate (LY) from HEK293T cells transiently transfected with plasmid constructs containing the truncated proteins LRP5N-WT_{9L}, LRP5N-p.E528_V529ins21 and LRP5N-Mut_{3L}. Immunodetection of membrane-marker beta-actin in cell lysates or by Coomassie staining of a 60-kDa protein in the conditioned medium were used as controls for comparable loading. Only LRP5N-WT_{9L} is strongly detected in conditioned medium, while the mutated constructs LRP5N-p.E528_V529ins21 and LRP5N-Mut_{3L} were found only in cell lysate indicating disturbed protein secretion.

Patient 4 was homozygous for common polymorphisms in *LRP5,* but no putative disease-causing mutations could be identified upon Sanger sequencing of *LRP5.* The PCR amplification of exon 4 was repeatedly unsuccessful in this patient despite several different primer combinations which produced normal amplification products in control samples. cDNA sequencing revealed the loss of exons 4 and 5 in the patient sample (Figure 10A). As exons 3, 5 and parts of intron 3 could be sequenced from the genomic DNA of Patient 4 (Figure 10B), the most probable explanation is a homozygous deletion involving exon 4 and part of the adjacent introns. The amplification of a PCR-fragment spanning the breakpoints was repeatedly unsuccessful.

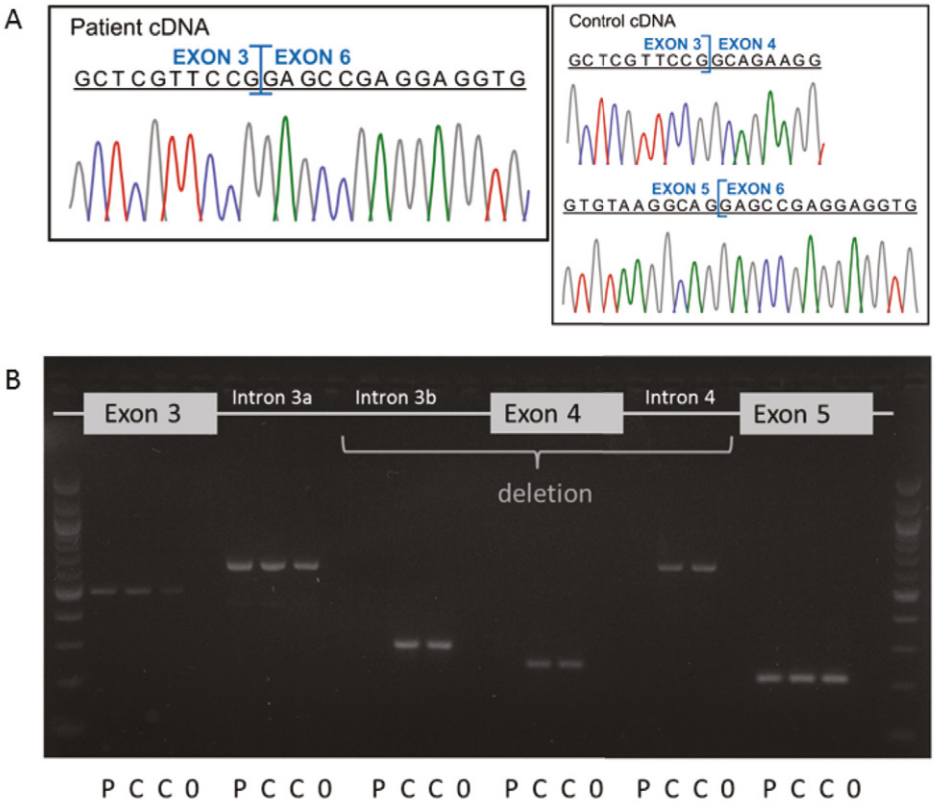


Figure 10. (A) Electropherograms of *LRP5* cDNA amplicons from Patient 4 and a control revealing the loss of exons 4 and 5. (B) PCR-amplified genomic DNA fragments from Patient 4 and controls visualized on a 2% ethidium bromide gel. All studied amplicons; exons 3, 4 and 5, areas of intron 3 (a: 2735-3671 nucleotides and b: 4198-4503 nucleotides downstream of exon 3) and intron 4 (607-1213 nucleotides downstream of exon 4) could be detected in two control samples (C) and are clean in water samples (0) serving as negative controls. PCR amplification was repeatedly unsuccessful from patient (P) DNA in the areas of intron 3b, exon 4 and intron 4, suggesting a homozygous deletion of exon 4 and adjacent intronic parts. Exon 5 and at minimum of 10 adjoining intronic nucleotides could be sequenced from both patient and control samples.

To conclude: The presented cases are patients with typical phenotypic features of OPPG. For this study, patients with mutations affecting the splicing of *LRP5* were selected. Prior to this study, only four splice site mutations in *LRP5* had been published (Ai *et al.*, 2005; Narumi *et al.*, 2010) and this was the first report evaluating the effect of human *LRP5* splice aberrations on the function of the respective LRP5 protein.

Two of the mutations in these patients affect immediate and highly conserved splice sites (+/- 2 bases from exons). Both substitutions, AG to GG of the splice acceptor site and GT to TT of the donor site, are common causes of exon skipping (Baralle *et al.*, 2008). Due to the lack of tissue samples and RNA from Patients 1 and 2, a heterologous splicing assay was used to confirm exon skipping and exclude activation of cryptic splice sites. For Patient 1, the loss of exon 20 removes the entire transmembranic domain of the encoded protein and hinders its function as a cell-surface signalling receptor. For Patient 2, the splicing out of exon 5 induces a premature stop codon before the first YWTD propeller in LRP5.

The splice site mutation c.1584+4A>T in Patient 3 resulted in the insertion of 21 amino acids into the second beta-propeller of the extracellular part of LRP5. (Figure 11) The same insertion has recently been found in a Japanese patient who carries a different mutation affecting the same splice site: c.1584+1G>A (Narumi *et al.*, 2010). Both these mutations inactivate the donor splice site, which activates the same cryptic splice site downstream. The insertion disturbs the intracellular transport of the protein thereby hindering its transit to the cell membrane and its function as a receptor.

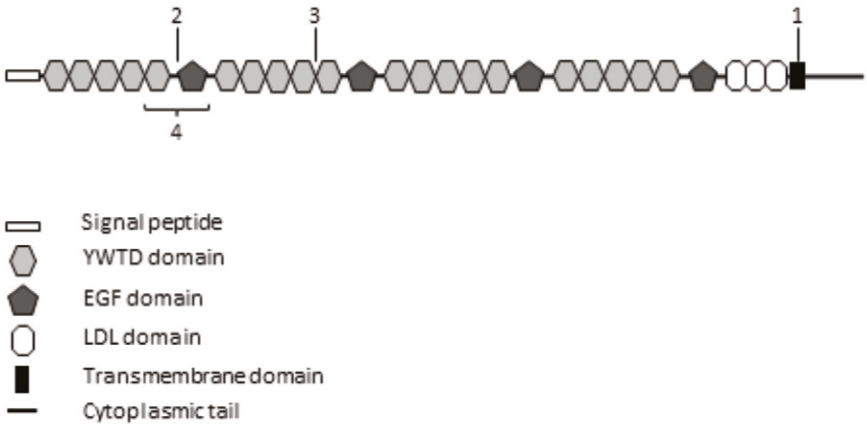


Figure 11. Schematic presentation of LRP5 and the areas affected by the mutations presented in this study. 1. The splicing out of exon 20 in Patient 1 removes the transmembrane domain of the protein. 2. The in-frame splicing out of exon 5 in Patient 2 induces a stop codon at the first EGF domain. 3. In Patient 3, the insertion of 21 amino acids after exon 7 affects the second β-propeller between two YTWD domains. 4. The loss of exons 4 and 5 in Patient 4 causes the loss of the last YWTD domain in the first β-propeller and the entire first EGF domain.

The fourth mutation was a deletion of exon 4 with parts of the adjoining introns causing the loss of exons 4 and 5 in the cDNA. The loss of exon 5 may be due to loss of splice enhancer elements in intron 4. This large deletion, which removes the first EGF domain and an adjacent YWTD domain in the extracellular domain of this receptor protein, is likely to significantly impair the protein function and the finding confirms the involvement of LRP5 in the patient's disorder.

The beneficial results of treatment with bisphosphonates in Patient 1 are in concordance with previous reports (Bayram *et al.*, 2006; Barros *et al*, 2008; Streeten *et al*., 2008). Low BMD was diagnosed in all six heterozygous carriers who underwent DXA; one had osteoporosis and five osteopenia. This supports previously published data indicating a dominant negative effect on BMD of heterozygous *LRP5* mutations (Saarinen *et al.*, 2010). One parent has adult-onset diabetes mellitus. Diabetes does not seem to be a common complication in OPPG, but studies on a large OPPG-family and a mouse model show over-representation of hyperglycemia through beta-cell dysfunction and of dyslipidemia (Fujino *et al.*, 2003; Saarinen *et al.*, 2010). Larger studies on heterozygous mutation carriers are necessary to explore the possible link between *LRP5* mutations and metabolic diseases.

Study III
Clinical and genetic findings in children with primary osteoporosis

The principal aim of this study was to better characterize primary non-OI osteoporosis in children; an increasingly recognized but poorly defined condition. This condition, also called idiopathic juvenile osteoporosis (IJO), has previously been described as a syndrome presenting around puberty with multiple fractures, vertebral compression fractures (VCF), and progressive kyphosis, bone pain, and anthalgic gait (Dent and Friedman, 1965; Smith, 1995) leading to a spontaneous recovery before adulthood. The age of onset is, however, more variable than previously thought. Many patients remain symptomatic in adulthood, some with severe sequelae from their osteoporosis (Smith, 1995).

Clinical findings

The 27 children included in this study presented with symptoms of their osteoporosis at age 3.3 to 15.6 years (median age 10.1 years), which is in concordance with the wide age variation at presentation from nine months to 13 years age published by Smith (1995). Age at first fracture ranged from 1.2 to 16 years. Fifteen patients had recurrent low-impact peripheral fractures (n>3), most commonly in the radius, tibia/fibula, or clavicle. The median number of peripheral fractures was 3.0 (range 0-10). Twelve patients had VCFs at first assessment and six patients with a normal spine at the first assessment had sus-

tained VCF at follow-up. Sixteen patients had a BMD Z-score below -2.0 (Table XI). No gender predominance was observed; however, females tended to be more severely affected. According to guidelines from the International Society for Clinical Densitometry, the diagnosis of osteoporosis requires the presence of both a clinically significant fracture history and low BMC or BMD (Baim *et al.*, 2008). Ten of our patients demonstrated a BMD Z-score greater than -2.0. However, their clinical picture was that of recurrent fractures and VCF.

As in previous studies, none of the biochemical markers of bone metabolism were diagnostic or indicative of disease severity. IJO has earlier been coupled to hypercalciuria, but only seven of our patients demonstrated mild hypercalciuria at study assessment (Urine calcium / creatinine ratio ≥ 0.7 mmol/mmol). No abnormalities in Ca, PTH, or ALP could be found in our cohort. Two patients were vitamin D deficient (<37.5 nmol/L) and two patients had IGF-1 levels below the normal range for age.

We recruited 60 family members from all 24 families of the index patients. BMD measurement was performed on 18 fathers (median age 41.6 years, range 31.3-57.6 years), 22 mothers (median age 40.7 years, range 32.1-53.9 years), 11 brothers (median age 13.4 years, range 7.1-17.5 years), and nine sisters (median age 9.3 years, range 7.4-16.8 years).

In contrast to earlier studies, a strong genetic component seemed to be evident in our cohort. Osteopenia and osteoporosis were common in both fathers and mothers (Table XI). Eight mothers and seven fathers had BMD values in the osteopenic range, and two fathers and one mother had a BMD T-score below -2.5, which is indicative for osteoporosis (Khan *et al.*, 2004; Kaufman *et al.*, 2008). In further support of heredity, there were three families with affected sibling pairs and at least one parent with low BMD. In the first family, the younger sister (1A) presented at age 11 with recurrent peripheral fractures and VCF, while her symptomless older brother (1B) was found to have a thoracic VCF and low BMD (Z-score -2.1) upon study assessment. The father had a history of recurrent fractures and both parents were osteopenic (BMD T-scores -2.2 and -2.0). In the second sibling pair, the older sister (10A) presented with an anthalgic gait. She had VCFs, a BMD Z-score of -1.5 but no history of peripheral fractures. Her younger brother (10B) presented with the same symptoms and several VCFs. The father had osteoporosis (BMD T-score -2.7). In the third family the brother (15B) presented at age 10 years with VCFs after a fall. His BMD Z-score was -2.0. At first assessment his sister's (15A) BMD Z-score was -1.9 and she had a history of one low impact fracture. On follow-up her Z-score was -2.1 and she had sustained another peripheral fracture. The father had osteoporosis (BMD T-score -2.5) and the mother was osteopenic.

At least one of the parents had osteopenia or osteoporosis in 13 out of 24 investigated families, while both parents had normal BMD in only six families. This indicates heredity of the bone phenotype. Mild OI can easily be mistaken for non-OI primary osteoporosis. In our material, 12 patients were found negative for mutations in type I collagen upon genetic screening and no patients had features of OI. The screened patients did not differ clinically from the patients with clinical exclusion of OI. The patients had no features of other known hereditary forms of osteoporosis (summarized in the literary review, Table III).

Table XI. Bone health characteristics at presentation in the study subjects and in the parents. Bone mineral density (BMD) was measured with DXA at the lumbar spine. Subjects named "A" and "B" are affected siblings. N/A, not available.

Subject	Gender	Age (y)	BMD Z-score	Peripheral fractures	Vertebral fractures	Mother's T-score	Father's T-score
1A	F	11.1	-1.3	3	Yes	-2.0	-2.2
1B	M	15.6	-2.1	2	No*		
2	M	6.5	-2.1	3	Yes	1.7	-1.4
3	M	5.0	-0.4	2	No*	-0.2	N/A
4	F	13.0	-0.6	9	Yes	1.0	0.3
5	M	5.7	-3.3	0	Yes	N/A	-1.7
6	M	10.9	-2.1	3	No	-0.4	4.8
7	F	11.6	-2.5	2	Yes	-0.7	N/A
8	F	4.0	-1.0	4	N/A	0.2	-0.7
9	F	13.0	-4.4	10	No	-0.6	-0.6
10A	F	8.9	-1.5	0	Yes	-0.7	-2.7
10B	M	3.3	N/A	0	Yes		
11	F	10.8	-3.2	1	No	-1.6	-0.9
12	M	7.3	-2.1	3	No	-0.8	-1.2
13	F	10.8	-1.5	3	Yes	-1.4	-1.2
14	F	7.1	-2.3	3	Yes	0.1	0.8
15A	F	4.5	-1.9**	1	No	-1.0	-2.5
15B	M	10.4	-2.0	2	Yes		
16	F	12.6	-3.1	0	Yes	-0.8	N/A
17	F	14.7	-1.9	4	No*	0.0	-0.7
18	M	6.7	-1.8	5	N/A*	-1.4	-0.1
19	M	5.3	-1.0	3	No*	-2.5	-1.6
20	M	12.7	-3.1	5	No*	-1.9	N/A
21	M	5.5	-0.7	4	No	0.3	N/A
22	F	13.2	-2.6	3	No	-1.1	-1.0
23	M	14.2	-2.2	2	Yes	N/A	N/A
24	M	14.1	-2.5	2	No	-2.4	-0.7

* **Vertebral fractures diagnosed at later follow-up**

** **The lumbar spine BMD Z-score was -2.1 at age 8 years.**

Genetic findings

No disease-causing mutations were found in the screened genes *LRP5, LRP6,* and *PTHLH*. In *LRP5,* all discovered sequence variants could be found in public databases (Table XII). The minor allele frequency of four polymorphisms (Q89R, V667M, N740N, and A1330V) was significantly higher in children with primary osteoporosis when compared to healthy controls: A1330V (27% in patients vs. 5% in controls, p>0.0001), Q89R (8% vs. 0.3%, p>0.0001), V667M (12% vs. 1%, p>0.0001), and N740N (31% vs. 6%, p>0.0001). The other LRP5 polymorphisms (p.Leu20dup, IVS4-4T>C (c.884-4T>C), F549F, E644E, V1119V, and R1188R) occurred at similar frequencies between patients and controls.

The *LRP5* variants, A1330V and V667M, decrease Wnt signaling *in vitro* (Kiel *et al.*, 2007; Urano *et al.*, 2009) and have repeatedly been associated with low peak bone mass, reduced BMD, and osteoporotic fractures in adult Caucasians (Brixen *et al.*, 2007; Ferrari *et al.*, 2004, 2005; Kiel *et al.*, 2007; Koay *et al.*, 2004; Koller *et al.*, 2005; Saarinen *et al.*, 2007; Richards *et al.*, 2008; van Meurs *et al.*, 2006, 2008). Each risk allele may affect the BMD by 2-5% (Kiel *et al.,* 2007; Urano *et al.*, 2009). N740N has been shown to associate with BMD in children, but in Caucasian adults the results have been contradictory (Ferrari *et al.*, 2004; Kiel *et al.*, 2007; Koay *et al.*, 2004, 2007). In Asians, Q89R and N740N associate with low BMD (Koh *et al.*, 2004; Lau *et al.*, 2005, 2006; Urano *et al.,* 2004; Zhang et al 2005), but only one study presented an association with A1330V in this population (Urano *et al.*, 2009).

The high frequency of the above mentioned polymorphisms among our patients may thus contribute to their skeletal phenotype, especially for e.g. Patient 1A, who is homozygous for the minor alleles in A1330V and N740N, and heterozygous for V667M. The other siblings with primary osteoporosis (10A and 10B, 15A and 15B), on the contrary, carried none of these variations in LRP5.

In ***LRP6***, all discovered polymorphisms (C308C, P448P, V483I, P955P, V1062I, and C1270C) were previously known and no difference in frequencies could be found between patients and controls. This supports previous findings of no association between variations in *LRP6* and BMD (van Meurs *et al.*, 2006, 2008).

The choice of ***PTHLH*** as a third candidate gene was mainly based on a murine low BMD phenotype which closely resembles that of juvenile primary osteoporosis (Miao *et al.*, 2005). No sequence variants in *PTHLH* were found in the study cohort, however.

Table XII. Genetic variations in *LRP5* and *LRP6* in patients with juvenile osteoporosis and in controls. Only one individual from each sibling pair has been included. The changes noted in bold were significantly more common in the patients than in control subjects.

Nucleotide change	Amino acid change	dbSNP #	Allele frequency (n, %)	
LRP5			**13 patients (26 alleles)**	**171 controls (342 alleles)**
c.58-61dupCTG	p.Leu20dup	rs72555376	dupCTG: 2 (8%)	dupCTG: 43 (13%)
c.226A>G	**Q89R**	rs41494349	G: 2 (8%)*	G: 1 (0.3%)
c.884-4T>C	-	rs314776	C: 12 (46%)	C: 99 (29%)
c.1647T>C	F549F	rs545382	C: 1 (4%)	C: 16 (5%)
c.1932G>A	E644E	rs2277268	A: 1 (4%)	A: 18 (5%)
c.1999G>A	**V667M**	rs4988321	A: 3 (12%)*	A: 2 (0.6%)
c.2220C>T	**N740N**	rs2306862	T: 8 (31%)*	T: 19 (6%)
c.3357A>G	V1119V	rs556442	G: 6 (23%)	G: 52 (15%)
c.3564G>A	R1188R	rs117289001	A: 1 (4%)	A: 5 (1%)
c.3989C>T	**A1330V**	rs3736228	T: 7 (27%)*	T: 18 (5%)
LRP6			**24 patients (52 alleles)**	**50 controls (100 alleles)**
c.924T>C	C308C	rs7978064	C: 2 (4%)	C: 5 (5%)
c.1344C>G	P448P	rs10082834	G: 1 (2%)	G: 6 (6%)
c.1447G>A	V483I	rs7975614	A: 1 (2%)	A: 1 (1%)
c.2865C>T	P955P	rs34143723	T: 1 (2%)	C: 0 (0%)
c.3184A>G	I1062V	rs2302685	G: 9 (17%)	G: 15 (15%)
c.3810C>T	C1270C	rs1012672	T: 1 (2%)	T: 5 (5%)

*p<0.0001 for comparison between patients and sequenced controls

To conclude: These findings indicate that children with primary non-OI osteoporosis have a variable pattern of age of onset, clinical severity, and inheritance. This disease entity is poorly known and the prognosis for untreated children varies greatly from complete recovery after puberty to progressive, debilitating osteoporosis according to the few reports available (Dent and Friedman, 1965; Smith, 1995). Genetic factors are likely to play a major role in the pathogenesis since the majority of the patients in the present cohort had at least one parent with low BMD. The discovered clustering of *LRP5* polymorphisms which affect Wnt-signaling may affect osteoporosis phenotypes, possibly in conjunction with polymorphisms in other genes affecting bone turnover. The polymorphisms in *LRP5* cannot alone, however, explain the osteoporosis seen in affected carriers, as the impact of each polymorphism on BMD has been estimated to only a few percent (Kiel *et al.*, 2007; Urano *et al.,*2009).

The polymorphisms found in *LRP6* were not over-represented in affected children. There is thus far only one published osteoporosis-causing mutation in *LRP6*, and the phenotype included early-onset coronary disease (Many *et al.*, 2007). GWAS studies have not found any link between *LRP6* polymorphisms and BMD, which is in concurrence with the results presented here (van Meurs *et al.*, 2006, 2008).

The choice of a third candidate gene, *PTHLH,* was based on similarities between the phenotype seen in the patients and in a mouse model harboring a *PTHLH* mutation (Miao *et al.*, 2005). The protein encoded by *PTHLH*, PTH-related peptide (PTHrP) is known to function locally in bone recruiting osteogenic cells, promoting bone formation and preventing apoptosis (Miao *et al.*, 2005), and a promoter polymorphism affecting its receptor, PTHR1*,* associates with variations in BMD and adult height in humans (Scillitani *et al.*, 2006). The *PTHLH* gene has not been implicated in GWAS studies focusing on BMD variations, nor were sequence variations found in patients in this study. This gene, however, remains interesting when screening for mutations in children with severe primary osteoporosis, due to that fact that it is extremely evolutionarily conserved. Only seven exonic variations with minor allele frequencies below 1:10000 have been found thus far (UCSC and NHLBI databases); variations cannot be a common cause of osteoporosis, but it is possible that these can be found in some osteoporosis subsets.

Study IV
Clinical and genetic findings in Calvarial doughnut lesions syndrome

This study focuses on a rare dominantly inherited condition of Calvarial doughnut lesions (CDL). The name depicts the circular translucencies surrounded by rings of sclerotic bone seen on the skull radiographs of affected patients. Other features include osteopenia, frequent peripheral and vertebral fractures, transient cranial nerve palsies, dental caries, jaw tumors, and deafness. S-ALP is often increased. Previous reports dating from 1969-2001 include 29 sporadic cases and three families comprising 22 cases (Baumgartner *et al*., 2001). The genetic background of this disorder is unknown. Three affected patients have been presented here, from a three-generation non-consanguineous family (Figure 12)

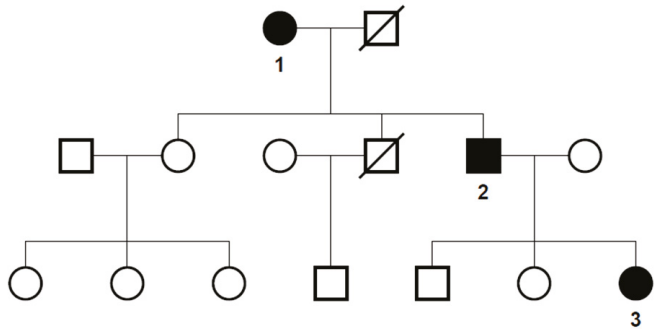


Figure 12. Pedigree of the family with Calvarial doughnut lesions. Affected individuals are marked in black, unaffected in white.

Clinical findings

The index patient is a girl, who was admitted for knee pain at the age of 2 years. She had no findings of inflammatory joint diseases and her spine radiograph revealed coarse trabeculation consistent with osteopenia. Her skull radiograph findings were normal in infancy. At the age of 3 she was diagnosed with congenital glaucoma, which has since been addressed with both surgical and maximal medical treatments. She has sustained long bone low-impact fractures (radius, age 6; femur, age 11). At age 11 her LS BMD Z-score was -1.9 and she was diagnosed with thoracic VCFs. She has no features of OI: her joint laxity, sclerae, teeth, and hearing are normal and she has no Wormian bones. Skull radiograph revealed sclerotic lesions around and anterior to the coronary suture (Figure 13). Her blood biochemistry was consistent with high turnover osteoporosis: S-ALP was high (454-992 U/L, reference range 115-435 U/L) and bone turnover markers were increased. All other markers of calcium metabolism were normal. Histomorphometric findings are presented below. She was referred to the Bone Clinic at Helsinki University Central Hospital at age 12. Intravenous dinatriumpamidronate was administered from age 13 on reduced dosage (1 mg/kg every 3 months) for one year followed by normal dosage (1 mg/kg on three consecutive days every 4 months) for two more years, after which treatment was discontinued. The treatment rendered some improvement to her vertebral changes but only little change in BMD. Bone turnover markers normalized during treatment and remained so at the one-year follow-up after discontinuation of treatment (Table XII). She has not sustained any new fractures and the changes in her vertebrae have remained stable.

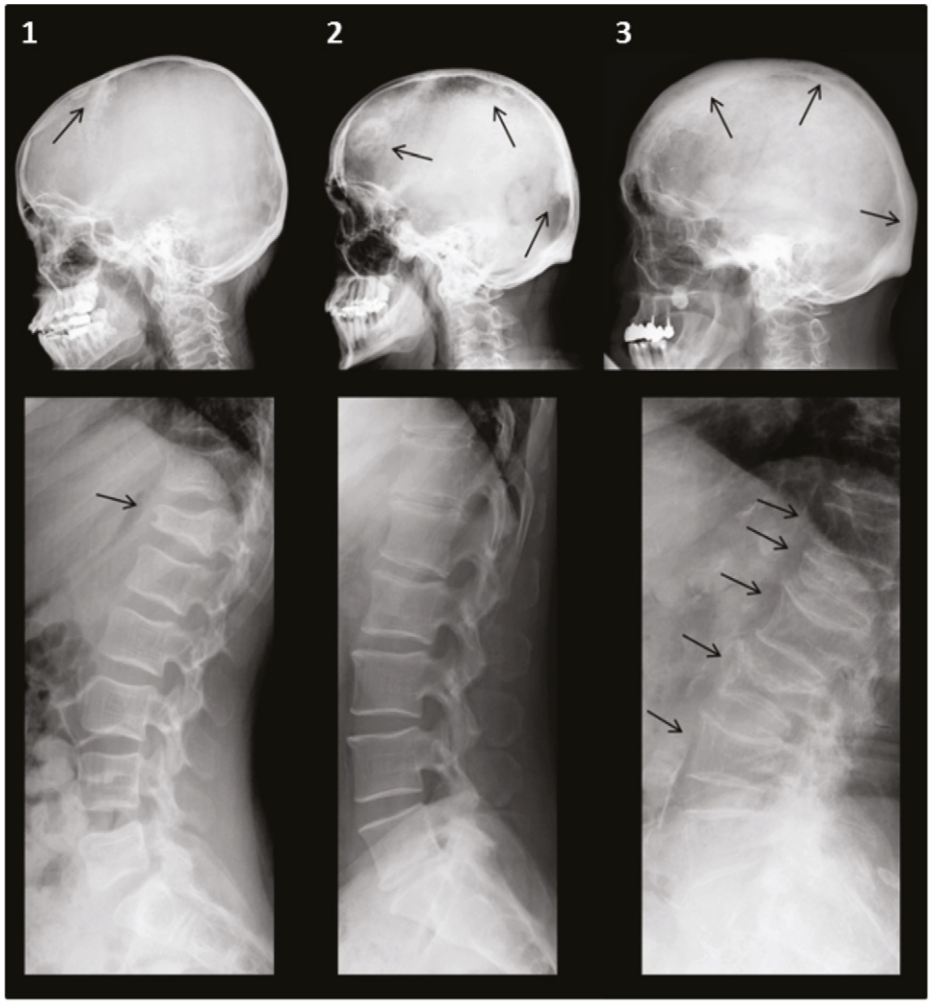


Figure 13. Skull and lumbar spine radiographs of the affected family members; changes are indicated with arrows. (1) The index patient has sclerosis around and anterior to the coronary suture and a thoracic VCF. (2) The father's radiograph reveals multiple CDLs. He has no obvious lumbar spine changes. (3) The paternal grandmother has generalized irregular hyperostosis of the skull and multiple severe lumbar VCFs.

Table XIII. Changes in bone mineral density and biochemical bone turnover markers with dinatriumpamidronate treatment in an adolescent with Calvarial doughnut lesions.

Age (y)	treatment stage	BMD Z-score			Bone turnover markers (reference value)					
		L1-4	hip	WB	P-ALP		S-PINP		U-INTP	
13	Pre-treatment	-1.4	-0.1	-0.7	534	(115-435)	1300	(400-800)	1221[a]	(330-377)
14	Pamidronate, reduced dosage 1 year	-1.3	-0.3	-0.4	158	(90-335)	148	(19-84)	249[a]	(268-377)
15	Pamidronate, normal dosage 1 year	-1.0	-0.3	-0.6	76	(80-210)	64.1	(19-84)	58[a]	(86-169)
16	Pamidronate, normal dosage 2 years	-0.8	-0.4	-0.8	80	(80-210)	46.2	(19-84)	55[a]	(55-71)
17	One year post-treatment	-0.7	-0.4	-0.5	81	(80-210)	53.0	(19-84)	58[a]	(49-58)

BMD, bone mineral density; WB, whole body P-ALP, plasma alkaline phosphatase, in U/L; S-PINP, serum N-terminal peptide of type I procollagen, in µg/L; U-INTP, urinary N-telopeptide of type I collagen, in nmol/mmol Cr. [a]reference values calculated based on sex and pubertal stage based on published data (Mora et al., 1998)

The **father** has a history of over 10 fractures to his skull, metacarpal and carpal bones, fibula, tibia, ulna, radius, and clavicle since the age of 4 years. At 7 years he was diagnosed with multiple thoracic VCFs. He has suffered from transient palsies on the left facial nerve at 41 years, to the right at 43 years, and to the right oculomotor and trochlear nerves at 49 years. His MRI and CT findings showed no narrowing of cranial nerve canals. He has no features of OI and his hearing is normal. His eye pressures are slightly elevated but he needs no glaucoma medication. Blood biochemistry analyses revealed normal levels of ALP, vitamin D, PTH, and Ca. At age 49 years his LS BMD T-score was -1.6 and CDLs were seen on a skull radiograph (Figure 13)

The **paternal grandmother** has sustained at least 14 peripheral fractures (humerus, radius, tibia, fibula, clavicle, pubic ramus, ribs) since childhood. She has multiple VCFs and diffuse irregular hyperostosis of the skull, but no CDLs. No BMD measurement has been performed. She was diagnosed with chronic congestive glaucoma at age 58, she receives medical treatment and has undergone bilateral argon laser trabeculoplasty at age 59 and cataract surgeries at 69 and 73 years of age. In addition, she has been diagnosed with goiter, hypertension, ischemic heart disease, chronic atrial fibrillation, asthma, and Alzheimer's disease.

Histomorphometric findings

Bone histomorphometry was assessed from an iliac crest sample from the index patient. The bone tissue was abnormal with thin trabeculae and an increased trabecular number. This finding explains her fracture susceptibility despite normal bone volume. There were no signs of fibrosis or sclerosis. Osteoclasts were abundant, which is consistent with our patient's high levels of biochemical bone turnover markers. Parameters of bone mineralization were normal. (Table XIV)

Table XIV. Histomorphometric parameters based on a bone biopsy sample from a 12-year old girl with Calvarial doughnut lesions. Reference values are presented as medians and range based on published pediatric reference data (Glorieux *et al*., 2000). Abnormal values are in bold.

Parameter		Result (reference)
BV/TV (%)	bone volume of tissue volume	21.63 (24.4 ± 4.3)
Tb.Th (µm)	**trabecular thickness**	**80.29 (148 ± 23)**
Tb.N (/mm)	**trabecular number**	**2.69 (1.66 ± 0.22)**
Tb.Sp (µm)	**trabecular separation**	**290.86 (464 ± 78)**
W.Th (µm)	wall thickness	44.36 (45.1 ± 6.9)
O.Th (µm)	**osteoid thickness**	**8.61 (6.7 ± 1.7)**
OS/BS (%)	**osteoid surface of bone surface**	**12.46 (22.1 ± 7.8)**
OV/BV (%)	osteoid volume of bone volume	1.63 (2.12 ± 1.00)
Ob.S/BS (%)	osteoblast surface of bone surface	6.38 (6.7 ± 4.5)
ES/BS (%)	erosion surface of bone surface	12.71 (14.9 ± 5.6)
Oc.S/BS (%)	**osteoclast surface of bone surface**	**3.66 (0.94 ± 0.38)**
MAR (µm/day)	mineral apposition rate	0.87 (0.87 ± 0.09)

Genetic findings

Collagen I genes *COL1A1* and *COL1A2* were sequenced from the index patients DNA sample due to the high-turnover osteoporosis, which also can be seen in collagenopathies. No mutations were discovered. Mutations in *LRP5* have been linked to both osteoporosis and an eye phenotype. Upon Sanger sequencing of all exons of *LRP5,* no putative disease-causing mutations in could be found in the patients DNA sample. The patient was heterozygous for a leucine repeat

variant (c.58_60dup /p.Leu20dup), which occurs in about 13% of the Finnish population (reference population result presented in Table XIII). The variation is not over-represented in cohorts with proneness to fractures. Due to the high frequency of the patient's variation in the general population and its modest effect on Wnt signaling, it is unlikely to cause the phenotype presented in our pedigree (Chung *et al*., 2009).

To conclude: According to these findings and previous reports, the rare syndrome of CDL seems to be of dominant inheritance. The genetic background of this disease is still obscure, and no mutations were found in *COL1A1, COL1A2,* and *LRP5.* In a previous report on one patient with CDL no mutations could be found in genes encoding collagen I, III, and V, and the conclusion was that CDL could be distinguished from OI by MRI of the skull and collagen analysis (Baumgartner *et al*., 2001).

The phenotype varies even among family members, which is a common feature of dominantly inherited diseases. Patients in this study had findings of osteopenia, recurrent peripheral and vertebral fractures in childhood, cranial nerve palsies in adulthood, and CDLs / sclerosis of the skull. They had no signs of hearing deficit or jaw tumors, as has been presented in earlier case reports, and this is the first known family with CDLs and glaucoma (Baumgartner *et al,* 2001). The reason for this discrepancy in phenotype may be that the causative genetic change resides in different genes affecting the same pathway. It is also possible that the glaucoma found in our pedigree is due to some other genetic defect unconnected to CDL.

The histomorphometric finding of high turnover osteoporosis in the index patient is consistent with earlier reports. Biopsy showed no signs of sclerosis or fibrous dysplasia, but the skull radiograph indicates that she has local sclerotic bone changes. High turnover osteoporosis in iliac crest bone biopsies and changes consistent with fibrous dysplasia from skull bone biopsies have been reported in patients with CDL, and some patients have had increased bone tissue sclerosis. (Baumgartner *et al*., 2001; Royen *et al*., 1974).

Dinatriumpamidronate was administered cyclically for three years due to the high-turnover bone metabolism and VCFs. The patient tolerated the medication well and achieved normalization of bone markers, some improvement in her vertebral changes but only a small amelioration of BMD with medication. This is the first report on response to bisphosphonates in a patient with CDLs. A post-treatment bone biopsy would be of interest to show whether the trabecular thickness improved with treatment.

6. STUDY LIMITATIONS

Due to the small number of patients in the studies, phenotypes were presented in a descriptive manner and statistical analyses on the data were underpowered in most cases.

In **Study I**, none of the family members were classified as "healthy" in the linkage analysis, as the onset and phenotype of a dominantly inherited disease can vary, and most subjects were rather young. This decision reduced the power of the two-point analysis and limited the statistical significance of the findings. The fact that the probands may have inherited the osteoporosis phenotype from either or both parents also complicated the analysis, and it was surprising that a genomic region shared by maternal relatives and both probands could not be found. The discovery of two putative linkage areas within the pedigree also made the study more challenging. Targeted next-generation sequencing was performed on three affected individuals only. Whole-exome sequencing of all affected individuals would of course have provided better data. The intronic and intergenic areas were not sequenced. Exon-flanking intronic regions and known CpG areas were screened to identify variations affecting splicing and gene expression, but to determine whether intronic variants are disease-causing the study would need to be complemented, with gene expression analyses for example. It can not be excluded that changes within the non-coding areas, and not the non-synonymous variations presented, could possibly cause the presented phenotype, nor can it be dismissed that some small genomic region in between the initial microsatellite markers may possibly contain the disease-causing variant. Regarding next-generation sequencing, there is always a risk of low coverage in certain genomic regions, especially in areas with a high content of C and G bases. Results from screened individuals were compared in pairs to search for variants which may have been missed in one individual, but if the sequencing failed in two or three screened individuals, some variants in the targeted regions may have been missed. No functional studies on the missense variants identified in this screening have yet been performed. Future analyses on gene function and on larger patient subsets are necessary before conclusions regarding a link between these variations and the phenotype can be drawn.

Studies II and III contained a selection of patients from international centers. These centers provide consultation services to a large population and due to geographic issues the patients or their family members were not always available for further analyses and sampling. Therefore the choice of methods for genetic analyses after the initial DNA screening had to be made based on sample availability in Study II. This difficulty also limited the amount of parental DNA available for screening in Study III. In Study II, the breakpoints of the deletion

in Patient 4 could not be pinpointed despite numerous sequencing attempts. The identification of the breakpoints could have provided interesting information regarding intronic regulatory elements in *LRP5*.

In Study IV the investigated family included only three affected family members. Only a candidate gene approach could be used, which failed to reveal the causative genetic defect in the family. Since CDL is an extremely rare disease, it could not be included with other cases in this study. The possibilities for genome-wide analyses to identify the causative mutation are further discussed in the following chapter.

7. GENERAL DISCUSSION AND FUTURE PROSPECTS

Despite the strong genetic background of osteoporosis, GWASs have thus far provided limited information regarding significant osteoporosis-causing genotypes. Replication rates are low between different studies and the numerous genes and SNPs linked to osteoporosis based on GWASs are estimated to contribute to only 1-4% of the variation in population BMD (Lau *et al*., 2006; Styrkarsdottir *et al*., 2008; Yerges *et al*., 2009). The approach taken in Study I was to focus on families with a high prevalence of severe osteoporosis as to enable the pinpointing of significant osteoporosis-causing gene mutations. No single causative mutation was discovered in affected individuals, but these results may indicate that the disease seen in this family is of complex inheritance. Common genetic variations impact bone strength and a combination of variations can cause a significant bone weakness phenotype. Two genomic regions were identified in this study, in chromosomes 1 and 11, which reached the threshold for suggestive linkage. Neither of these areas have previously been linked to osteoporosis. Based on the targeted next-generation sequencing, three non-synonymous variations in *BCAR3, MMP10* and *DYNC2H1* were identified. Only the variation in *BCAR3* was predicted to be deleterious to protein function, and this gene has no known link to bone phenotypes thus far. The variant identified in *MMP10* has previously been implicated to have a role in cartilage and ligament maintenance (Kevorkian *et al*., 2004; Posthumus *et al*., 2011), but the phenotype in our patients does not indicate defective cartilage function. Biallelic mutations in *DYNC2H1* cause the rare polydactyly-short-rib syndrome (Hoey *et al.*, 2011). The encoded protein is involved in the function of mechanosensory cilia in the osteocytes, and could be a possible candidate gene for further studies. The impact of the identified variations should be elucidated in future studies.

In contrast to earlier reports referring to idiopathic juvenile osteoporosis as a sporadically occurring disorder, the results of Study III implicate a strong heredity of low BMD phenotypes, since many of the assessed children had at least one parent with low BMD (Dent and Friedman, 1965; Smith, 1995). Certain common SNPs in *LRP5* were also shown to be over-represented in children with bone weakness. The results support the impact of common variations in this Wnt pathway receptor on bone integrity. Study patients probably carry other genetic variations which, in combination with the SNPs in *LRP5*, induce the presented phenotypes, as the SNPs in *LRP5* only have modest effects on BMD according to earlier studies (Styrkarsdottir *et al.*, 2008). Mutations in *LRP5*, on the other hand, can cause significant bone phenotypes, and Study II offered the first published functional data on splice site mutations in this gene. The Wnt pathway affects many aspects of metabolism. There are results linking the Wnt pathway to the presence of diabetes, hypercholesterolemia, and vascular disease (Saarinen *et al.*, 2009). The patients described here were too young to

exhibit such changes, but exploration of this pathway may, in years to come, explain the clinically known correlation between osteoporosis and metabolic diseases.

In Study IV, the knowledge of the rare CDL syndrome was expanded. No known large-scale genetic screening has been performed on patients with CDL previously. Based on the local findings of fibrous dysplasia, one putative candidate gene could be *GNAS*, which encodes the stimulatory G-protein α-subunit of the G-protein complex. Activating mutations in *GNAS* are known to cause McCune-Albright syndrome and fibrous dysplasia (Lee *et al.*, 2012). If a candidate gene screening failed to identify putative mutations, genome-wide screening methods could be applied for mutation identification. As this disease seems to be of monogenic inheritance, microsatellite linkage analysis would be the logical method of choice. This would require international collaboration, since this study pedigree is too small and hitherto published cases are scarce. There is also the risk that separate pedigrees carry mutations in different genes of a mutual pathway, which would render the use of linkage analysis more complicated. Whole-exome sequencing would identify putative disease-causing exonic changes, but since the disease is suspected to be of heterozygous inheritance, sorting the large amount of data achieved by this approach would cause significant challenges (Ng *et al.*, 2008). Another approach would be gene expression analyses, preferably from material derived from patients' skeletal tissue. Based on our small pedigree, the search would be truly challenging using currently available screening methods.

8. CONCLUSIONS

1. Genetic variation (SNPs) in *LRP5* affects bone health already in childhood and adolescence. *LRP5* and possibly other genes involved in the Wnt-signalling pathway are likely to have a major role in bone mass development and maintenance of skeletal integrity from early childhood. Screening for harmful sequence variations in these genes may provide a tool for early detection of individuals with an increased risk for symptomatic osteoporosis. Such markers would be helpful in optimal targeting of preventive measures.

2. CDL is not caused by mutations in *COL1A1*, *COL1A2*, or *LRP5*. The disorder thus represents a distinctive form of dominantly inherited primary osteoporosis. The histomorphometric findings are characterized by increased bone turnover. Identification of the causative genetic defect requires a next-generation sequencing approach of larger cohorts of affected individuals but would be valuable in understanding the pathophysiology of CDL and more widely, the pathogenesis of high-turnover osteoporosis.

3. We present a new form of inherited early-onset osteoporosis in a large Finnish family. Despite apparent autosomal dominant inheritance and an inheritance pattern similar to monogenic diseases, no genetic defect common to all affected subjects could be identified. Dominantly inherited diseases have reduced penetrance and this may have affected our analyses. Our findings indicate that genetic factors play a major role in primary osteoporosis and early clinical and radiographic screening of potentially affected individuals is recommended for early intervention to prevent severe spinal complications.

4. Variations in *BCAR3, MMP10,* or *DYNC2H1* may contribute to bone weakness phenotypes. The role of these genes and the functional implications of the identified sequence variations need to be further elucidated in future studies.

The search for new osteoporosis-causing genes is of importance, as the discoveries help to resolve the mechanisms causing the molecular and cellular pathogenesis of the disease. The findings may aid in the development of new therapeutic agents - as was the case with denosumab, which is a monoclonal antibody which binds to RANKL thereby hindering osteoclast proliferation and bone resorption (Cummings *et al.*, 2009). The significance of the Wnt pathway in bone building was unveiled by the identification of mutations in *LRP5* by linkage analysis in patients with high and low BMD phenotypes (Gong *et al.*, 2001; Little *et al.*, 2001). The use of lithium, an old and well-known drug for bi-

polar disorder, is now investigated as an osteoporosis treatment due to its ability to activate the Wnt pathway (Mosekilde *et al.*, 2011). The other advantage of new genetic findings is the possibility to screen patients at risk for genomic variations. As our knowledge regarding the clinical impact of these variations increases, genetic screening may become a tool for early patient identification, prevention, and targeting of treatments.

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